



CFlow User Guide

Science is hard. Flow cytometry should be easy.®

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Chapter 1 The Basics - Operating the Accuri® C6 Flow Cytometer®

Introduction to CFlow and Running the C6

The C6 Flow Cytometer system includes CFlow or CFlow Plus software for controlling the instrument, acquiring data, generating statistics, and analyzing results. Throughout this User Guide, CFlow refers to both CFlow and CFlow Plus unless indicated otherwise.

CFlow Workspace

The CFlow workspace provides access to all the functions needed to acquire and analyze data. CFlow is divided into three separate tabs: **Collect**, **Analyze** and **Statistics**. This section focuses on data acquisition and the **Collect** tab.

The **Collect** tab appears when CFlow is launched and is used to acquire data from samples processed on the C6. The **Collect** tab enables physical control of the C6, provides access to settings needed to specify the threshold and stop criteria as well as many analytic functions.

Figure 1-1 shows a partial **Collect** tab. The Sample Grid is laid out in the configuration of a 96-well plate to facilitate both organization of experiments and data acquisition from sample tubes. CFlow acquires each sample into its own well in the Sample Grid. The wells can be filled with data in any order that is convenient. White wells do not contain data and are available to use for data collection. Blue wells contain data. A red outline around a well indicates that well is currently selected for viewing or collecting data.

Instrument Controls

The left-hand column of the CFlow **Collect** tab provides access to all instrument controls needed to collect data. Before data collection can begin, the Traffic Light below the Sample grid must be green and the words “**C6 Cytometer is connected and ready**” appear in the message box.

Getting Started

This section provides a step-by-step guide for running validation beads. The validation will demonstrate many of CFlow features and ensure the C6 is working properly before running experimental samples. All the individual features of CFlow are described in detail in Chapter 4.

Complete the initial instrument set-up as outlined in the C6 Flow Cytometer Instrument Manual, Section 4, before continuing.

Starting CFlow

Open CFlow by double clicking on the CFlow icon on the desktop. The message box in CFlow will say **Cytometer Not Connected**.

C6 Startup Procedure

1. Check sheath bottle (blue ring) fluid level and fill if needed. Accuri recommends the use of 0.2 µm filtered, deionized, H₂O with bacteriostat added (Accuri Part# KR-220). CFlow will alert the user if the bottles need attention when the C6 is turned on and CFlow is running.
2. Empty the waste tank (red ring).
3. Check Cleaner (green ring) and Decontamination Solution (yellow ring) bottles. Fill if necessary.

Refer to C6 Flow Cytometer Instrument Manual, Section 4.3.

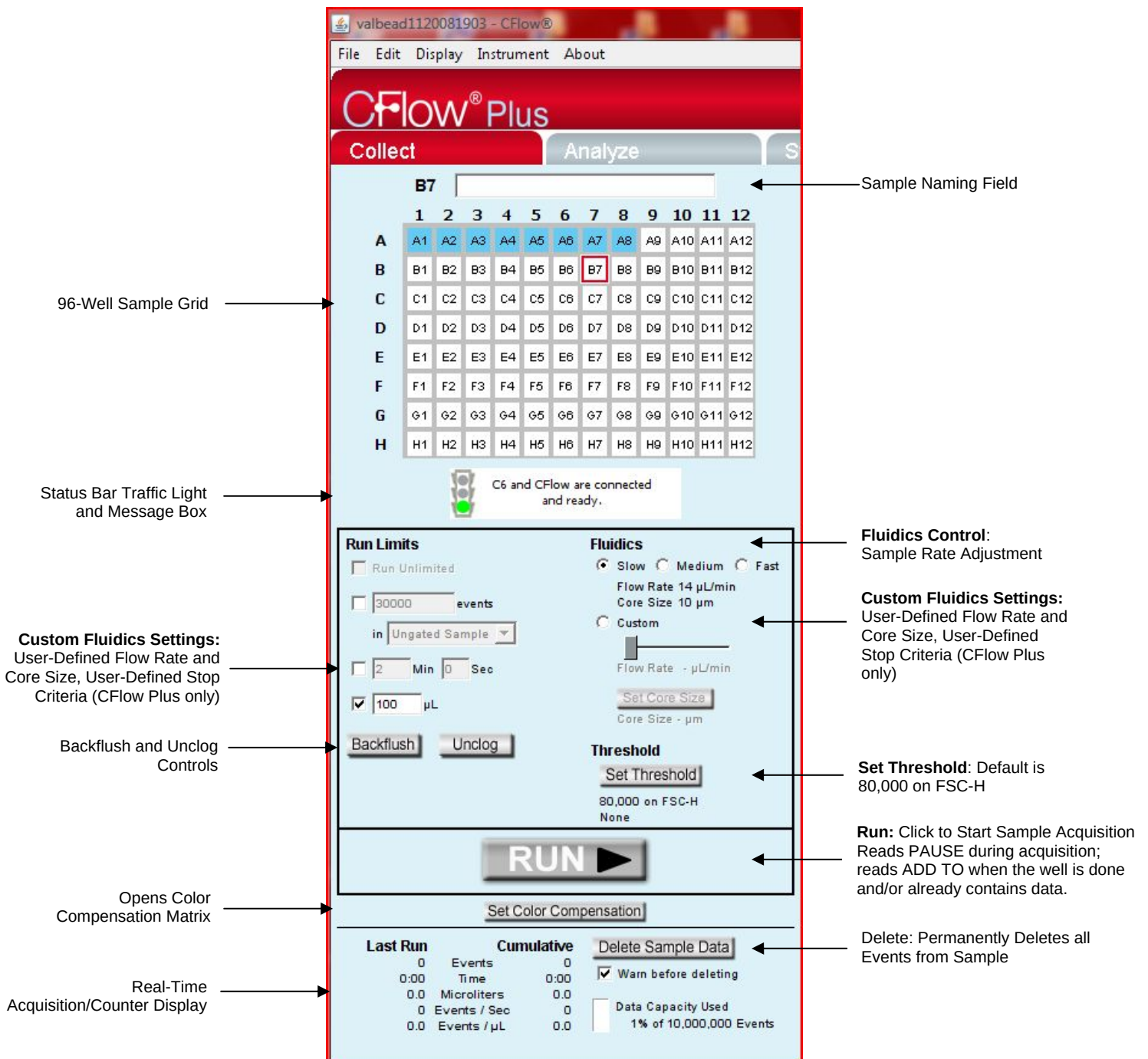


Figure 1-1 The CFlow Control Panel in detail

- Replace the tube on Sample Introduction Probe (SIP) with a fresh tube containing at least 2 mL filtered, deionized H₂O.
- Firmly press the On/Off button on the front of the unit to turn on the C6. A blue light will flash and then stay illuminated. After ~20 seconds, the CFlow traffic light will show yellow. After ~60 seconds, the C6 pumps will start to run. When powered on, the C6 will automatically flush the fluidics lines with sheath. This process takes approximately 3 minutes.
- When the C6 is ready for data collection, the CFlow traffic light will change to green. Begin data

collection when the Traffic Light is green and the C6 Status Message Box reads **C6 and CFlow are connected and ready**.

For optimal performance, the C6 should be on for at least 15 minutes prior to processing samples.

C6 Improper Shut Down Recovery Message

If the message shown in Figure 1-2 is seen at startup, the C6 will take approximately 6 minutes to recover and return to the green-light, ready state, as opposed to the usual 3 minute startup procedure. This may occur on initial C6 startup when the unit is first received. It will also occur after an interruption of power to the unit.

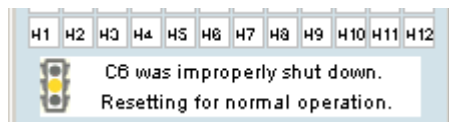


Figure 1-2 C6 Improper Shut Down Recovery Message

Running Validation Beads

Accuri provides an initial set of pre-diluted validation beads. Store the beads at 4°C in the dark. The beads expire within 2 weeks of receipt. Refer to **Reagents Required** for ordering information.

Validate the performance of the C6 with validation beads each day that the cytometer is used. The C6 should be able to distinguish a defined number of peaks within each fluorescence channel. If these peaks are not discernable there may be a problem and any data collected may be flawed.

It is important to collect and save a bead data file every day that data is collected. The user can easily address many C6 issues with assistance from Accuri Technical Support. If a problem is experienced that requires telephone assistance, Technical Support may ask for data from the bead file to assist in troubleshooting.

Reagents Required

1. Spherotech 8-Peak Validation Beads (Accuri Part# QA-100) or the prediluted 12x75 mm tube from Accuri labeled 8-Peak Beads
2. Spherotech 6-Peak Validation Beads (Accuri Part# QA-110) or the prediluted 12x75 mm tube from Accuri labeled 6-Peak Beads
3. Sheath fluid: Deionized, filtered water (0.2 µm filter) plus Bacteriostatic Concentration Solution (Accuri Part# KR-220)

Running the Bead Samples

1. Be sure that the C6 has been switched on for at least 15 minutes.
2. Verify that the file named **"Bead Template.c6t"** has been copied to the computer attached to the C6. This file is on the CFlow CD shipped with the C6 and also available on the Accuri website.
3. Double-click on the bead template file to open or load the file from the CFlow **File** Menu.
4. Place an empty 12x75 mm tube on the SIP.
5. Click on "BACKFLUSH".
6. Place a fresh tube with 2 mL filtered, deionized H₂O on the SIP.
7. Click on well A1 to select it. Well A1 should now have a red box around it.
8. Deselect the **Run Unlimited** check box under **Run Limits** in the Instrument Control Panel.

9. Select the time check box (**Min Sec**) in the Instrument Control Panel and input 2 minutes.
10. In the Control Fluidics section of the Control Panel, select **Slow**. The flow rate should display 14µL/min and **Core Size** should display 10 µm.
11. Click on the **Threshold** button to set the Threshold to 80,000 on FSC-H (default).
12. Press **Run**. While data is being acquired, well A1 will flash blue.
13. Click **Pause** then the **Delete Sample Data** button in the Control Panel of the CFlow screen to clear data collected during the run. The data well should change from blue to white and the counters will reset.
14. Deselect the time check box and click the **Events** check box. Verify that it is set to 50,000 events in **Ungated Sample**. If not, enter 50,000 events and select the **Ungated Sample** check box.
15. Replace the tube on the SIP with a tube containing suspended 8-peak beads. Vortex the tube prior to placing it on the C6.
16. Click **RUN** to start acquisition. Acquisition will automatically stop after 50,000 total events are acquired. Do not click on **ADD TO**. **ADD TO** is used to add more data to a well that already contains data. The R1 region may not encompass the main population of bead events on the FSC-H vs. SSC-H plot. This will be addressed in **Analyzing and Recording Your Validation Bead Data**.
17. Name this sample including the date processed in the text box just above the Sample Grid. Samples can be named at any time; before, during, or after collection.
18. Remove the beads tube and wipe off the end of the SIP with a Kimwipe, or comparable material, to minimize bead carryover. To further minimize carryover, click **Backflush** while holding a reservoir or empty tube under the SIP. Fluid should drip or stream out of the SIP.
19. Place a tube with suspended 6-peak beads on the SIP. Vortex the tube prior to use.
20. Click on well E1 to advance to well E1. Verify that the **Events** check box is still selected and set to 50,000 events in **Ungated Sample**.
21. Click **RUN**. The R2 region on the FSC-H vs. SSC-H plot in the second row of the CFlow bead file may not encompass the main population of beads.
22. Name this sample including the current date in the text box just above the Sample Grid.
23. Remove the beads tube from the SIP and wipe the end of the SIP.
24. Place a tube with 2 mL of filtered, deionized H₂O on the SIP and advance to any empty data well.
25. Select the time check box (**Min Sec**) in the Instrument Control Panel and set it for 2 minutes. Click **RUN**.
26. Leave the tube on the SIP after the C6 stops running.

Always end each data collection session on the C6 by running filtered, deionized H₂O for 2 minutes. Leave this tube on the SIP between sessions and when shutting off the C6.

Saving the Data

Saving data as a CFlow file will save the CFlow workspace in its entirety, including collected sample data, plot layouts, gating, color compensation, and threshold settings that have been added. Changes made in the **Collect**, **Analysis** or **Statistics** tabs will be saved in a single comprehensive (and often large) data file. Data collected on the C6 should always be saved as a CFlow file.

There is no need to export after every sample run. Data from individual sample wells can be exported from CFlow as FCS 3.0 files at any time.

Save Your Data as a CFlow File

1. In CFlow, under the **File** menu select **Save CFlow File** (Figure 1-3) and save the bead data file as a "Validation Bead File". Use this file each day to collect Validation Bead data, advancing to a new well in row A or E for 8-peak and 6-peak beads, respectively. Include the date in the Sample Naming Field when naming each day's bead sample.

CAUTION: CFlow does not automatically save CFlow files to the computer hard drive. The data is saved locally in RAM until it is saved as a CFlow file or exported as FCS files. CFlow allows switching between data wells to view data even when data is not stored to the hard drive.

2. End every session of data collection on the C6 by placing a tube containing 2 mL of filtered, deionized H₂O on the SIP. **Run** for 2 minutes in an empty data well to clear the last sample's residue from the SIP. Leave the tube on the SIP after the run has finished.

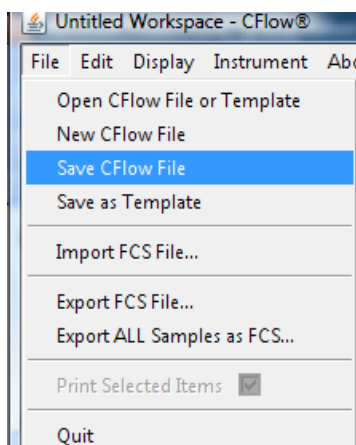


Figure 1-3 Saving a CFlow file

Analyzing and Recording your Validation Bead Data

Analyze the bead data using the **Collect** tab of CFlow.

1. Click on the well in the Bead File which contains the 8-peak bead data to select it (will be A1 if following previous procedure).
2. On the first FSC-H vs. SSC-H plot (scatter plot) in the bead file, adjust the pre-drawn region R1 to encompass the main population by clicking on the region (it will have thicker lines when selected) and dragging it so it looks like Plot 1 in Figure 1-4. This should contain 75-85% of all events in R1. There is usually a "shadow" population slightly higher in FSC-H than the main cluster of beads; this is normal for these particular beads (bead doublets or clumps). Do not include this group in R1.
3. View the next 3 plots - FL1-H, FL2-H, and FL3-H. They should be gated on the scatter region, R1. The phrase "**R1 in all**" should be visible next to the gray "**GATE**" button at the top of each of these 3 fluorescence plots (Figure 1-5). If not, click the **GATE** button and select the option "**R1 on all events**" from the pop-up gating list.
4. Position the predrawn horizontal **Marker Region** around the top (brightest, far-right) peak on each of the 3 fluorescence plots.

Use the **Zoom Tool**, located in the lower right corner of each plot (the button with the magnifying glass), to zoom in on the top peak. Then, adjust the marker tightly around the peak. Click on the **Expand Tool** (the button with a square and four arrows) to zoom back out.

5. Compare the bead run to the manufacturing results sent with the C6. If the C6 is performing properly, data should look similar to that in Figure 1-6 for the 8-peak beads. There should be one main

population of beads on FSC-H vs. SSC-H, 8 discernable peaks on FL1-H and FL2-H, and 6 peaks on FL3-H. FL4-H performance will be checked with the 6-peak beads in a later step.

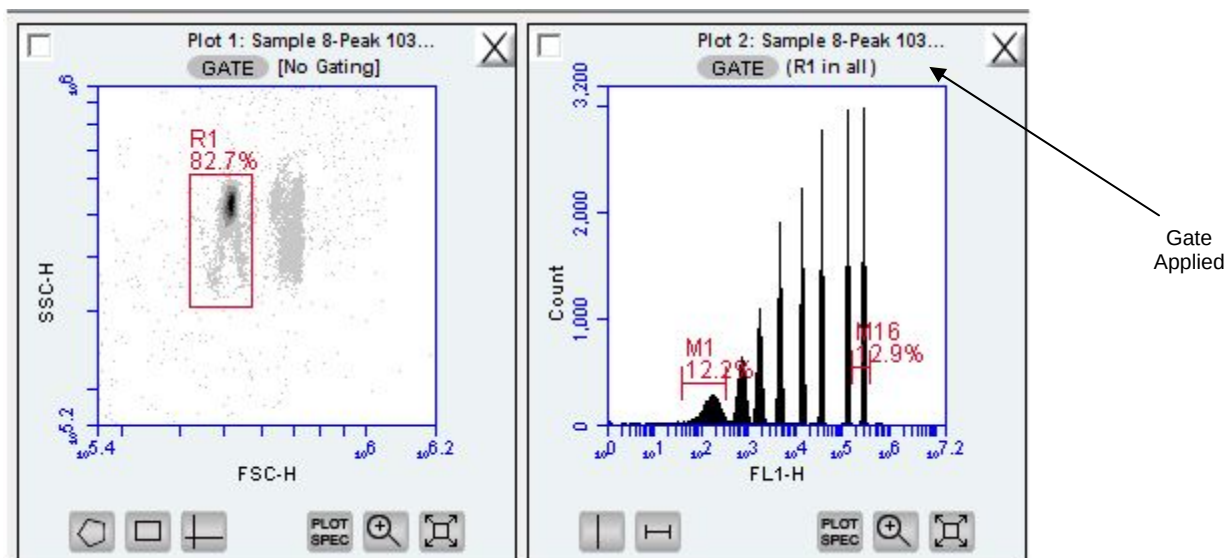


Figure 1-4 Proper position of R1 on Plot 1 and Gate selection for Plot 2

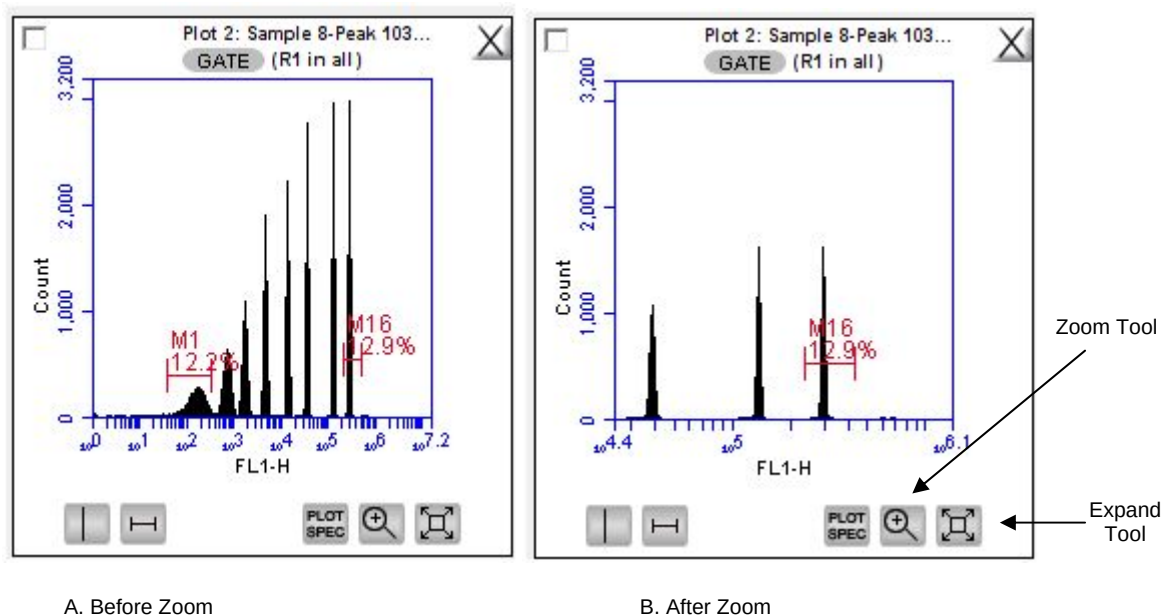


Figure 1-5 Location of Zoom Tool and Expand Tool, and effect of Zoom

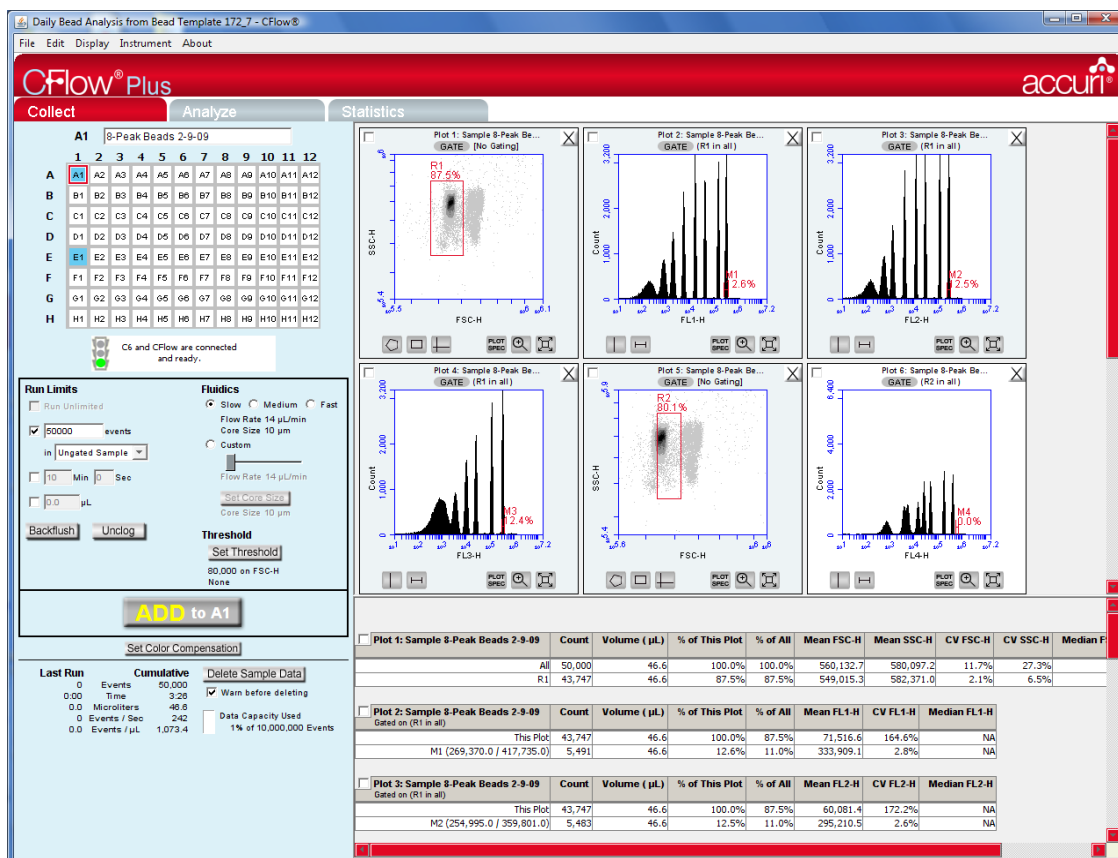


Figure 1-6 Example of 8-peak bead data collected with a C6. The number of expected peaks is: FL1-H (8), FL2-H (8) and FL3-H (6). The peaks in FL4-H are not relevant.

6. Select the well containing the 6-peak bead data (should be well E1).
7. Adjust the pre-drawn region R2 in the second FSC-H vs. SSC-H to encompass the main data peak similar to the procedure for the 8-peak beads. This population should look like an exclamation mark. The R2 region should encompass both major populations (Figure 1-7).
8. Confirm that the FL4-H plot is gated on the region R2. If not, click on the “GATE” button and select the option “R2 on all events” from the pop-up menu.
9. Adjust the **Marker** in the FL4-H plot to be tightly placed around the top (brightest) peak. Refer to Figure 1-7 for an example of good 6-peak bead data on a C6.
10. Record the number of peaks, the Mean channel numbers and CVs for the top peaks, and for the forward scatter of the beads for each parameter in the Accuri Cytometer Log. Additional copies can be found on the CFlow CD and on the Accuri website.

Please send a copy of the bead file, from the first time beads are run on the C6 after installation, to Accuri Technical Support: dmack@accuricytometers.com.

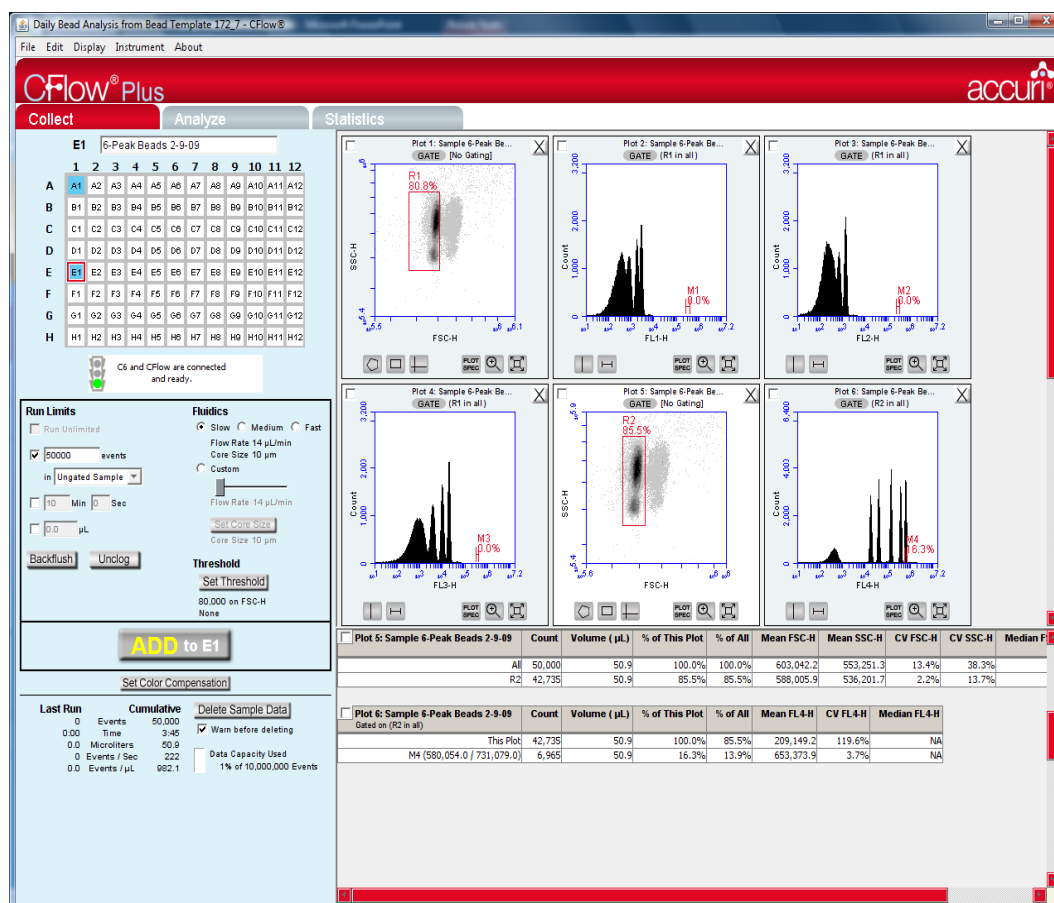


Figure 1-7 Example of 6-peak SPHERO APC Calibration Particles, shown in FL4-H. The peaks in FL1-H, FL2-H and FL3-H are not relevant.

Using the Statistics Tab in CFlow to Generate a Table of Mean Peak Channel and CV values

A convenient way to monitor the Validation Bead data, and thus the C6 performance, is to store the 8- and 6- peak validation bead data in a single CFlow file, saving the data from each day in its own well. Then, using the Statistics tab, a table can be created of the Mean channel numbers and CVs for the top peaks, and for the forward scatter of the beads to make it easy to determine if the C6 performance is stable.

In Figure 1-8, the C6 in question had been performing with a FSC-H CV of between 2% and 2.5% for over a month using the SPHERO Rainbow Calibration Particles. However, on the last day shown, the FSC-H CV had increased substantially as had the top peak CVs for FL1-H and FL2-H. This could indicate either bead degradation or a slightly dirty flow cell. Refer to Troubleshooting for possible fixes.

Also refer to Troubleshooting if any of the following conditions are experienced:

1. Very broad or multiple peaks for FSC-H on the 8- or 6-peak beads, excluding the shadow doublet population, or
2. Fewer than 8 peaks visible on FL1-H and/or FL2-H, or fewer than 6 peaks on FL3-H, or
3. Brightest peak CVs are >5.0% in any channel.

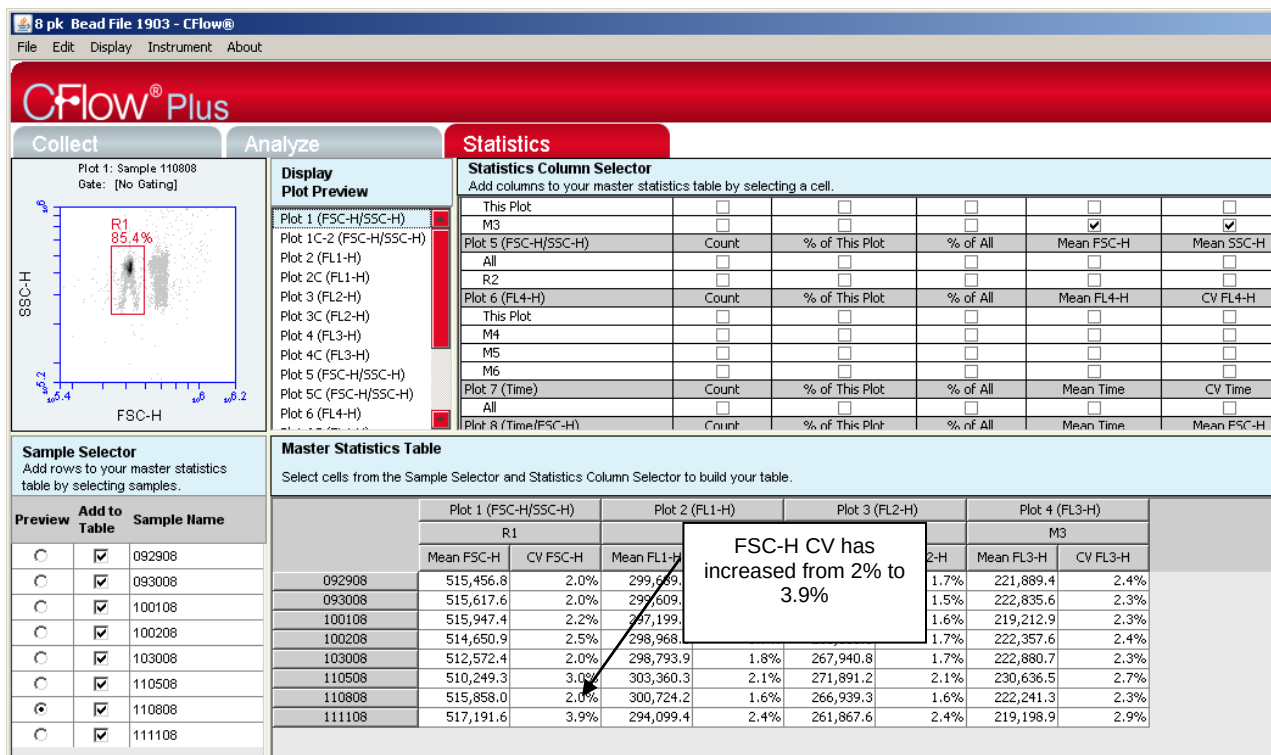


Figure 1-8 Statistics tab Display of 8-peak bead data from successive days

Troubleshooting

If there are very broad or multiple peaks for FSC-H, fewer than 8 peaks visible on FL1-H and/or FL2-H, fewer than 6 peaks on FL3-H or the brightest peak CVs are >5.0% in any channel check the following:

1. If bead data was acquired using the **Slow** rate (Under Fluidics Control in the Control Panel). If not, select Slow, re-suspend the beads by vortexing or flicking the tube, and recollect the data.
2. The age of the beads. If the beads have been diluted for more than 2 weeks, kept at room temperature or warmer, or exposed to light for long periods of time, their performance may be degraded. Make up new bead suspensions and run the bead sample again.
3. If there is a bubble or clog in the flow cell.
 - a. Run the bead sample again.
 - b. Remove the tube from the SIP, place an empty tube under the SIP and click **Unclog**. When the Unclog cycle is finished (Traffic Light will be green), run the bead sample again.
 - c. Remove the tube from the SIP, place an empty tube under the SIP and click **Backflush**. When the cycle is finished (Traffic Light will be green), run the bead sample again.

For further troubleshooting, refer to FAQs at www.AccuriCytometers.com or call Accuri Technical Support at 1.734.994.8000.

Chapter 2 Getting the Most from the C6

Materials Needed to Operate and Maintain the C6

- Sheath fluid: Deionized, filtered water (0.2 µm filter) plus Bacteriostatic Concentration Solution (Accuri Part# KR-220)
- Decontamination Solution: 10X Concentrate (Accuri Part # KR-200)
- Cleaning Solution: 10X Concentrate (Accuri Part# KR-210)
- C6 Flow Cytometer Maintenance Kits which include replacement Fluidic Bottle Filters (Accuri Part# CP-130 and CP-135), In-line Sheath Filters (Accuri Part# CP-140) and Peristaltic Pump Tubing (Accuri Part# CP-105)
- Validation beads (refer to page 7)

System Cleaning and Validation

Following a few simple rules ensures C6 optimal performance.

Keep the C6 Clean

- Always follow the startup and shut down procedures outlined in the C6 Flow Cytometer Instrument Manual in order to keep the SIP and flow cell clean, and the fluidics system free of air bubbles. Decontamination fluid is run through the C6 fluidics system at each shut down. This helps to keep the SIP and flow cell free of debris and protein build-up but may not prevent build-up from occurring.
- Always leave a tube containing at least 2 mL of filtered, deionized H₂O on the SIP in between uses, and at shut down.
- Replace the following User Serviceable Parts of the C6 **every two months**.
 - o Sheath bottle filter
 - o Decontamination and Cleaning solution bottle filters
 - o In-line Sheath filter inside the C6
 - o Peristaltic pump tubing
- Refer to the C6 Flow Cytometer Instrument Manual, Section 5, for replacement procedures and Section 6 for order information.

Keep the C6 Validated

- Run the Accuri-recommended Validation Beads each day the C6 is used. Refer to Chapter 1 for detailed instructions on Validation Beads.
- If CFlow Plus is installed on the C6 and volumetric or absolute cell counts are required, it is important that the system is accurately measuring the volume aspirated from each sample.
 - o Replace the peristaltic pump tubing every 2 months.
 - o Ensure that the SIP and flow cell are clean, and the fluidics lines are free of air bubbles. Refer to C6 Flow Cytometer Instrument Manual, Section 5.4b, Running the Cleaning Cycle and 5.5, Inspection of the Fluidics Lines, for directions.
 - o Validate volume measurement using Accuri's Volumetric Validation Beads or other counting bead product. Refer to C6 Flow Cytometer Instrument Manual.

Standard Operating Procedures for the C6

The following are recommended standard operating procedures.

Instrument Startup

1. Check fluid levels in all tanks and attend to them if needed.
2. Press the Power Button on the front of the C6 and open CFlow Software.
3. Wait until the Traffic Light in CFlow is green and message reads: **C6 and CFlow are connected and ready.**
4. Place an empty 12x75 mm tube on the SIP.
5. Click **BACKFLUSH** button in CFlow. A small amount of sheath fluid should appear in the tube.
6. Place a fresh tube containing 2 mL of filtered, deionized H₂O on the SIP.
7. In CFlow, Deselect the **Run Unlimited** check box and select the **Time** check box. Enter 2 minutes.
8. Click **RUN**. Wait for run to finish.
9. Click **DELETE EVENTS FROM SAMPLE**.
10. Keep the tube with water on the SIP until experimental samples are ready to be analyzed.

Care After Running Samples

Always perform this process after the last sample to ensure cells or other particles are not left in the SIP.

1. Place a tube with 2 mL of Cleaning Solution on the SIP.
2. Select an empty data well.
3. Select the **Time** check box. Enter 2 minutes.
4. Click **RUN**. Wait for run to finish.
5. Remove the tube of Cleaning Solution, and place a tube of 2 mL filtered, deionized H₂O on the SIP.
6. Select the **Time** check box and enter 2 minutes.
7. Click **RUN**.
8. When run is finished, leave the tube of water on the SIP.

Instrument Shut Down

1. Perform Care After Running Samples procedure detailed above.
2. Press the Power Button on the front of the C6.
3. When the Power Off process is completed the CFlow Message Box will read **C6 Cytometer not connected.**

Validation of Instrument Performance

Follow the directions in Chapter 1 for running the 8- and 6- peak SPHERO Calibration Particles. Use the Accuri C6 Cytometer Log, (spreadsheet located on the CFlow Installation CD) to record top peak CVs and Mean channel numbers for each fluorescence detector.

Calibrating the Fluidics System for Precise Volume Measurements

The C6 fluidics system is calibrated for a sample volume of 400 µL in a standard 12x75 mm tube during manufacture. Accuri suggests that you recalibrate your system for your most common tube type and volume to increase the accuracy of volume measurements. If the C6 is being used routinely for volume analysis,

perform volume calibration once a week. For intermittent volume analysis, perform volume calibration prior to each set of experiments requiring it. Volume measurements can only be performed in CFlow Plus software.

To ensure the most accurate volume measurements:

- The peristaltic pump tubing must be less than two months old.
- Run samples at Medium or Fast speeds.
- Calibrate the C6 to an equivalent sample volume/fluid height in tube as the sample to be measured.
- Never perform calibration with less than 150µL in any type of sample tube.

To Calibrate the C6

1. Place a tube of the same kind to be used in the experiment and containing a volume of sheath fluid close to the sample volume in the experiment + 65 µL on the SIP. For example, if cell samples are diluted to 400 µL in 12x75 mm tubes, then the Fluidics Calibration should be performed with a 12x75 mm tube containing 465 µL of sheath. The minimum sample volume to calibrate a 12x75 mm tube is 150 µL (run calibrate with 215 µL in the sample tube).
2. Under the **Instrument** Menu, choose **Calibrate fluidics**. The fluidics calibration will take ~ 5 minutes.

If calibration fails, the C6 will revert to its previous calibration automatically and will operate normally. However, the volumes recorded will not be optimized for the new sample volume. If more precise measurement is essential, try calibrating again.

Routine Instrument Maintenance

Part Number	Description	Replacement Interval
CP-102	Replacement SIP Guard	
CP-105	Peristaltic Pump Tubing (Set of 2)	Every 2 Months
CP-130	Sheath Bottle Filters (Large) (Pk of 5)	Every 2 Months
CP-135	Cleaner / Decon Bottle Filters (Small) (Pk of 5)	Every 2 Months
CP-140	In-line Sheath Filters (2 ea.)	Every 2 Months
CP-158	C6 Flow Cytometer Maintenance Kit (1 year supply of Peristaltic Tubing (CP-105), Bottle Filters (CP-130 and CP-135) and In-line Sheath Filters (CP-140))	Every 2 months
KR-200	Decontamination Concentrate Solution (10x)	As Needed
KR-210	Cleaning Concentrate Solution (10x)	As Needed
KR-220	Bacteriostatic Concentration Solution (For Sheath Fluid) (10 vials)	As Needed

Visit www.AccuriCytometers.com for a complete list of available parts and reagents.

Tips on Data Collection and Analysis with the C6

The Accuri C6 is a fixed voltage system.

There are no voltage or amplifier gain settings to adjust. The detector settings for light scatter and fluorescence are optimized and balanced during assembly. The Zoom Tool can be used to magnify an area of interest. In Figure 2-1, the Zoom Tool has been used to focus on the lymphocyte light scatter (Figure 2-1a) and surface marker fluorescence (Figure 2-1b).

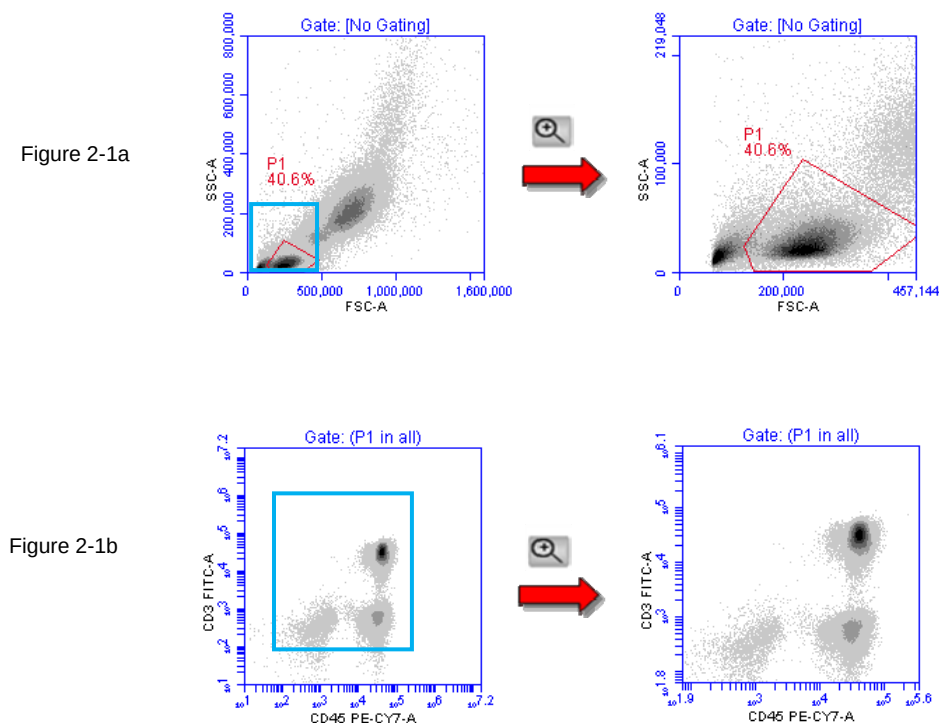


Figure 2-1 Zoom tool used to focus on lymphocyte light scatter and surface marker fluorescence

Fluorescence compensation can be set during or after data collection.

Data collection on the C6 is digital. This means that fluorescence compensation, used to adjust the spectral overlap from one fluorochrome to another, can be applied or adjusted, even after data has been collected and saved in a CFlow file.

Each data well in holds a maximum of 1 million events.

Data can be added to a well, even one already containing events, at any time, up to a total of 1 million events. When a data well already contains data, the well will be colored blue and the **RUN** button will read **ADD TO**.

Chapter 3 Using the Collect Tab

Introduction

The **Collect** tab appears when CFlow is launched and is the only tab which allows control of the C6. The **Collect** tab is used to set data collection thresholds and gates, stop criteria, and to control the fluidics, including sample collection using the RUN and PAUSE buttons. **Collect** also allows access to most of the analytic functions in CFlow including the creation of complex gates, setting fluorescence compensation, and generating statistics.

In this chapter, a four-color analysis of human peripheral blood will be used to illustrate many of the **Collect** tab functions, including:

- Acquiring data on the C6
- Creating plots (histogram, dot or density) in which to view data
- Setting stop criteria and thresholds
- Using regions and markers to create gates and obtain statistics
- Saving and printing plots and data

A CFlow file containing the data used in the following example, "HPB 4 Color Tutorial.c6", is available from the Accuri website, www.AccuriCytometers.com, and can be used as a tutorial tool. For the example shown here, the C6 has already been started-up and instrument performance validated.

The steps outlined here follow a workflow process where the C6 user is setting and applying gates, opening plots, and applying fluorescence compensation values in real time as data is being collected.

An alternative approach is to collect all data using minimal C6 set-up, with only the appropriate Thresholds, Fluidics rate, and Run Limits set initially. The data for all samples is then collected in quick succession, and analysis performed at a later time, including setting of gates and applying fluorescence compensation.

Experimental Design of Tutorial File

Four tubes are used to assess the CD3⁺CD4⁺ and CD3⁺CD8⁺ cell populations in the example cell analysis. These samples were prepared by staining peripheral blood with directly conjugated antibodies, followed by red cell lysis, according to standard methods.

	1	2	3	4
FITC	Isotype	Isotype	CD3	CD3
PE	Isotype	Isotype	Isotype	CD4
PE-Cy7	Isotype	CD45	CD45	CD45
APC	Isotype	Isotype	Isotype	CD8

Table 3-1 Experimental design for analysis of CD3⁺CD4⁺ and CD3⁺CD8⁺ cell populations in human peripheral blood. Samples 1 and 2 are the background control and the white blood cell gating control, respectively. Sample 3 is the control required for determining the percent of CD4⁺ and CD8⁺ cells within the CD45⁺CD3⁺ subset.

This experimental design does not contain single-stained fluorescence controls. Based on previous experiments, it is known that the only channels where fluorescence bleedover introduces ambiguity for setting gates are FITC bleedover into PE, and PE-Cy7 bleedover into the FITC and PE channels. Therefore, these samples are sufficient for this tutorial.

Refer to Accuri's online Fluorescence Compensation Tutorial for a more complete discussion of

fluorescence bleedover issues in flow cytometry and the impact it has on experimental design.

Opening a New CFlow Workspace and Collecting a Data File

1. Double-click the CFlow software icon on the desktop or, if CFlow is already open, select **File, New CFlow File** from the CFlow file drop-down menu.
2. The Default template is shown in Figure 3-1. Note that only a density plot of linear FSC-A vs. linear SSC-A is displayed. The plot is already “zoomed” to show channels 0 to 1,600,000 on FSC-A and 0 to 800,000 on SSC-A. Click on the **Plot Spec Tool** (lower right hand corner of Plot) to change the axes channel range, or to view axes on a logarithmic scale (Figure 3-2).

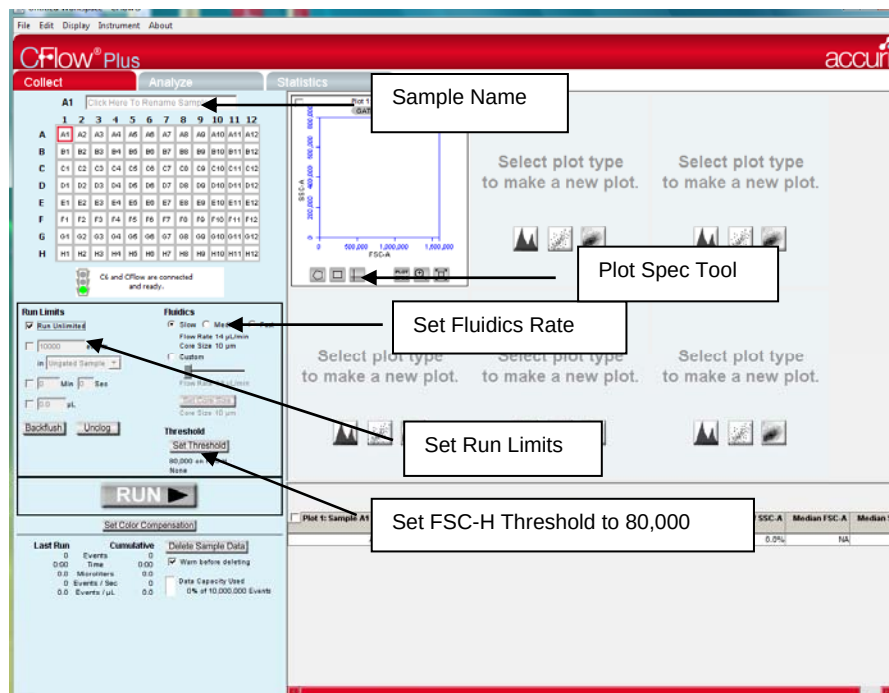


Figure 3-1 CFlow Workspace after selecting File, New CFlow File from the CFlow file menu

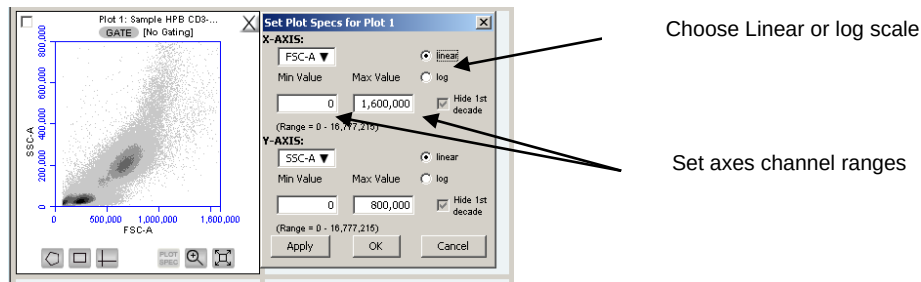


Figure 3-2 The Plot Spec Dialog Box. Changing these settings only affects the view of the data. No data is changed in, or lost from, the data file.

3. The Traffic Light below the 96-well grid in the upper left hand corner should be green with the message **C6 and CFlow are connected and ready**.
4. When CFlow is opened, sample well A1 will automatically be selected, as indicated by a red border.
5. Name the current sample by typing into the text box above the 96-well grid. Naming samples is optional, and can be done at any time. If no text is added, the sample will be named according to the

well location, e.g. "Sample A1".

6. Set a **Run Limit**. For the first sample, a good starting point is to collect 100,000 - 200,000 ungated, total events. The **Run Limit** can be changed once a gate has been set on the population of interest. Additionally, data acquisition can be stopped at any time or volume limit. Multiple run limits can be chosen; the C6 will stop on the first limit reached.
7. Set the **Fluidics Rate** to **Slow**. The maximum data rate that the system can accommodate is 10,000 events per second. To ensure the best data resolution, **it is recommended that samples be acquired at a rate of 5,000 events per second or less**, when possible. Once the data rate has been determined the setting can be adjusted to Medium or Fast if necessary.
8. The default value for thresholds is 80,000. If it is not already set at this value, set the **Primary Threshold** to channel 80,000 on FSC-H (Figure 3-3). Click on the **Set Threshold** button within the Fluidics control area, and a dialog box will open. Set the **Primary Threshold** on FSC-H to 80,000, and **Apply to All Samples**. This threshold should work well to gate out debris and noise from cell samples freshly isolated from human or mouse tissues.

A lower FSC-H threshold may be needed to effectively analyze small cells such as platelets or bacteria. A higher FSC-H threshold may be needed when working with large cells such as cell lines. The threshold settings can be raised or lowered at any time during analysis. If raising a threshold setting will result in data loss, CFlow will give a warning (Figure 3-4).

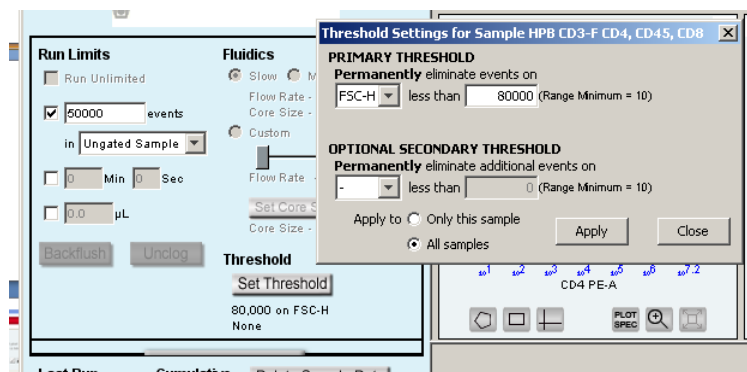


Figure 3-3 Threshold Settings dialog box

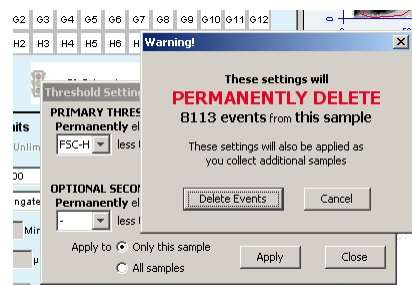


Figure 3-4 Warning message when raising a threshold value will result in data loss.

9. Before collecting the first sample, place an empty sample tube on the SIP and click the **Backflush** button (lower left side of CFlow template). This will clear any residue from the SIP.
10. Gently re-suspend the cells in the sample tube and place on the SIP.
11. Click the **RUN** button to start sample collection. The green Traffic Light will turn yellow and the message will read **Preparing to analyze sample**.
12. Once the fluidics initialization is complete, the Traffic Light will turn back to green and the message

will read **C6 is collecting data**. Well A1 will flash blue during data collection.

13. After the run limit is reached, well A1 will stop flashing and remain blue, indicating that the well contains data. The **RUN** button will now read **ADD TO** (Figure 3-5). If desired, more data can be collected into well A1 by clicking on the **ADD TO** button. Even after collecting samples in other wells, any well containing data can be returned to, and more events collected, at a later time.

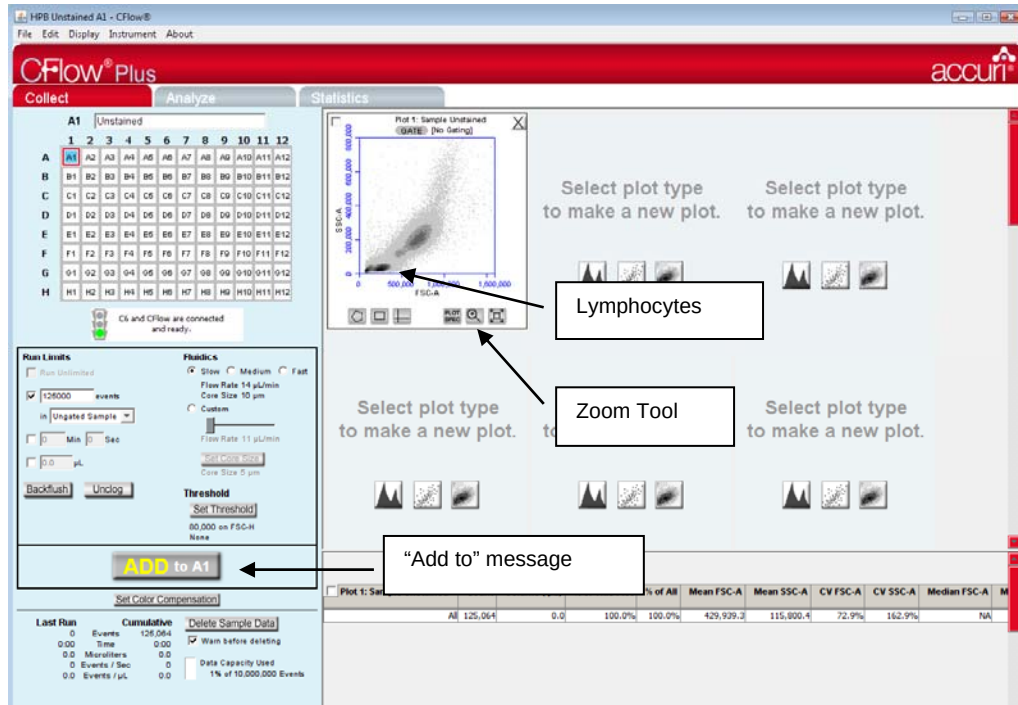


Figure 3-5 CFlow template after collection of Sample A1, human peripheral blood

Creating Plots and Applying Gates

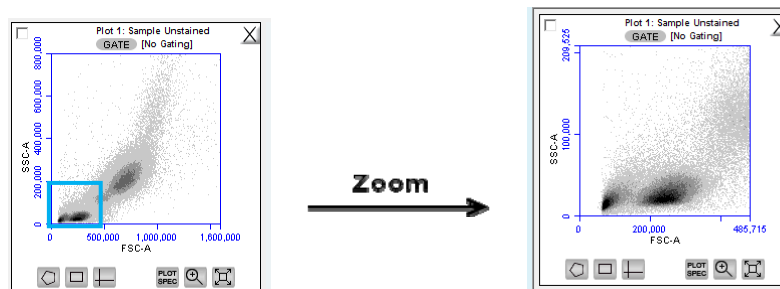


Figure 3-6 Use of Zoom Tool for a “close-up” view of the lymphocyte population in a human peripheral blood sample.

2. **Use the Zoom Tool to expand the view of the lymphocyte population.** Click on the **Zoom Tool** button (the symbol will turn blue) and use it to encompass the area on which to zoom (Figure 3-6).
3. Choose the **Polygonal** gating tool and draw a region, P1 (Figure 3-7). Left-click on the mouse to anchor each vertice; double-click to close the polygon. The percentage of cells within P1 for that plot appears on the screen automatically. There are two other options available for drawing gating regions, the **Rectilinear** and **Quadrant** gating tools. These tools will be used in other steps of this tutorial.

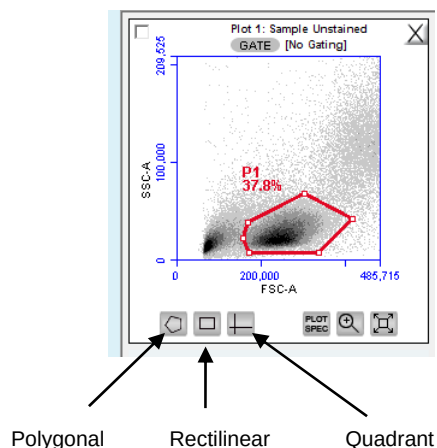


Figure 3-7 Polygonal gating tool was used to draw P1 on the 2D plot above. Other available gating tools are Rectilinear and Quadrant.

3. Open a new plot to display the data collected for FL1 and FL2. This data is currently stored in RAM. Select the **Density** plot icon from one of the empty plot corrals and a default FSC-A vs. SSC-A plot appears (Figure 3-8). There are two other plot options available, allowing the creation of a **Histogram** or a **Dot** plot.

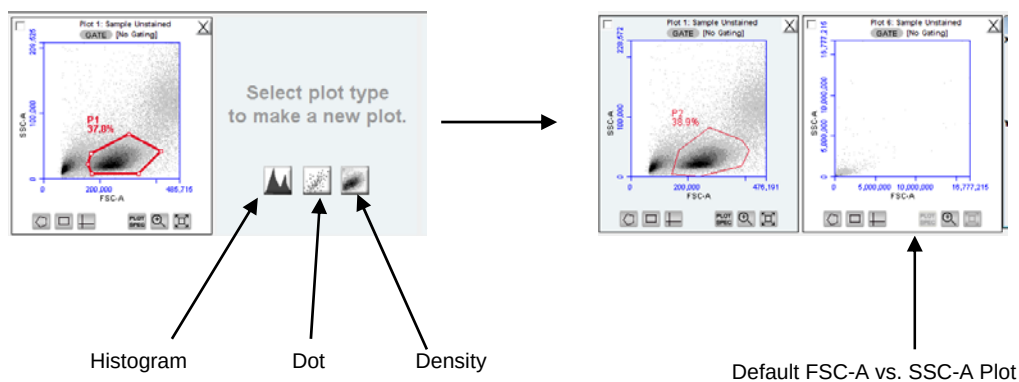
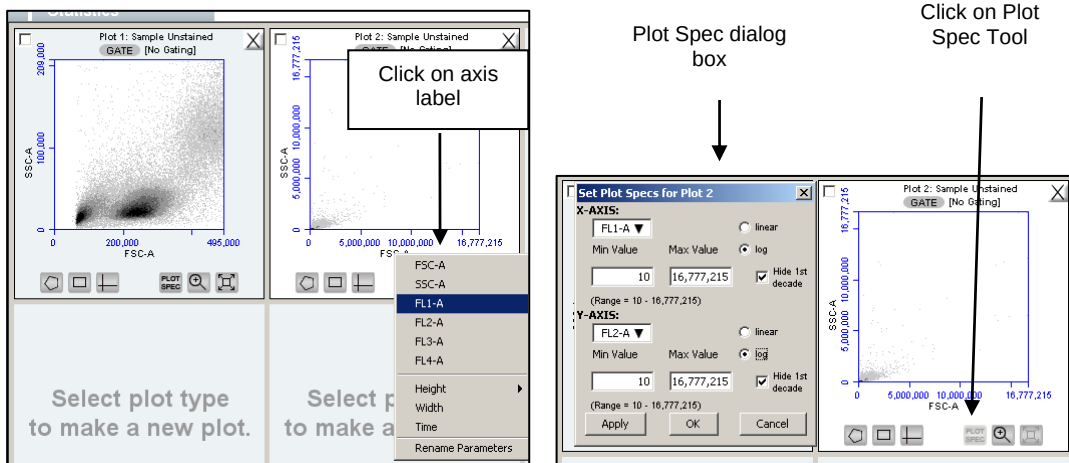


Figure 3-8 Opening a Density plot defaults to a linear FSC-A vs. SSC-A plot.

4. Change the newly opened linear FSC-A vs. SSC-A plot to a logarithmic FL1-A vs. FL2-A plot. There are 2 ways to accomplish this:
 - a. Click on the **Axis label** of the plot. A menu will appear, allowing the selection of the desired parameter to display. By default, fluorescence parameters are displayed logarithmically while light scatter parameters are displayed linearly (Figure 3-9 a).
 - b. Click on the **Plot Spec Tool**, select the appropriate parameter from the X-axis and Y-axis menus, and change the radio button selection from linear to log display (Figure 3-9 b).



a. Parameter axis menu

b. Plot Spec dialog box

Figure 3-9 Changing density plot axes parameters and scaling

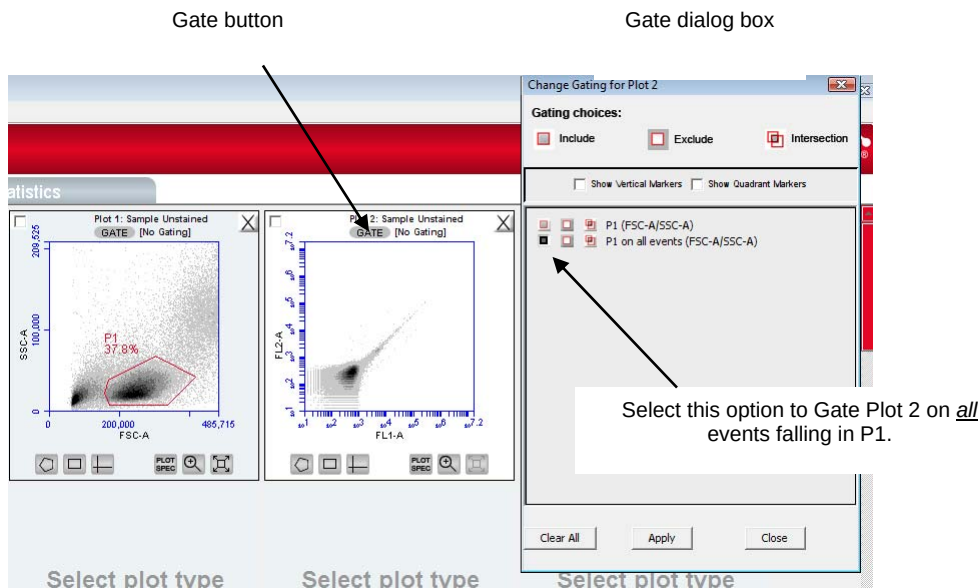


Figure 3-10 Selecting a gating option, which includes all events from a given gate, i.e. Plot 2 is now gated to Include "P1 on all events".

5. Apply a gate to the FL1-A vs. FL2-A plot. Click on the gray **GATE** button at the top of the plot to open the gating dialog box (Figure 3-10).
6. Select the gating option **Include P1 on all events** in the Gate dialog box and click **Apply** at the bottom of the box. The resulting gated Plot 2 is shown in Figure 3-11. Note that the text next to the gray **Gate** button will now read **(P1 in all)** instead of **(No Gating)**.

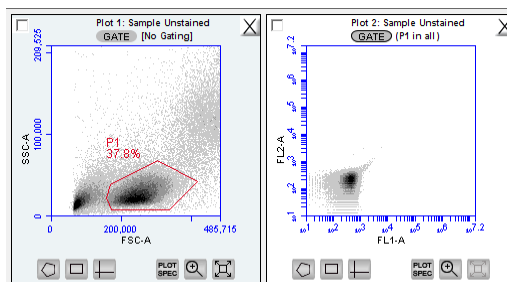


Figure 3-11 Plot 2 has been gated to include P1 (all events).

The events within any gate or series of nested gates can be selected to be included or excluded from a plot. In addition, gating combinations that do not appear as options in the Gating dialog box can be created by selecting the intersection choice (Figure 3-12).

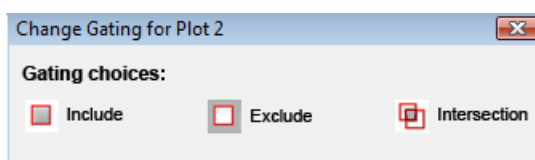


Figure 3-12 Gating choice options for plots: Include, Exclude, and Intersection.

Only Polygon (P), Rectilinear (R), and Marker (M) gating regions that have been drawn will appear automatically in the Gating dialog box list of options. To use Vertical or Quadrant markers for gating, select the appropriate check box in the Gating dialog box and those choices will appear (Figure 3-13).

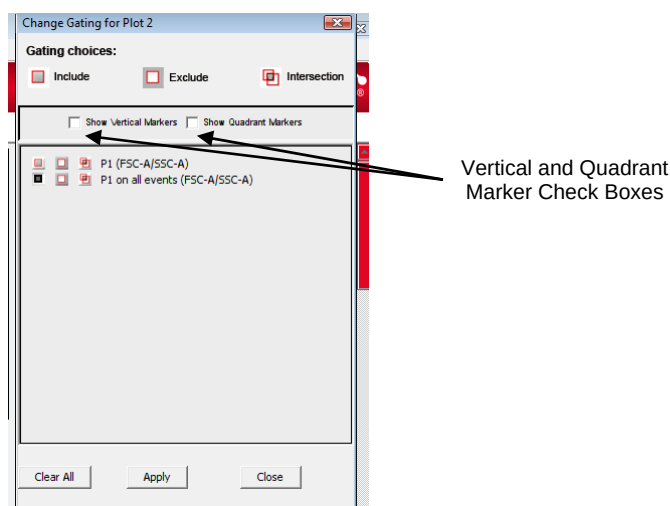


Figure 3-13 Adding Vertical or Quadrant Markers to the Gating list.

The initial display of any parameter on a logarithmic scale will be automatically zoomed to a scale that runs from channel 10 to 16.7×10^6 . For the majority of analyses, there are very few events which will fall into channels 0 to 10. Pre-zooming of the plots will save time by reducing the number of zoom steps. However, care must be taken when setting Markers (M), Regions (R) or Polygons (P) that require inclusion of channels lower than 10 on a pre-zoomed plot. It is recommended that users unhide the first decade of data, especially when setting fluorescence compensation, as this will prevent events from being excluded from gating regions. Refer to Figure 3-14a and b, for directions.

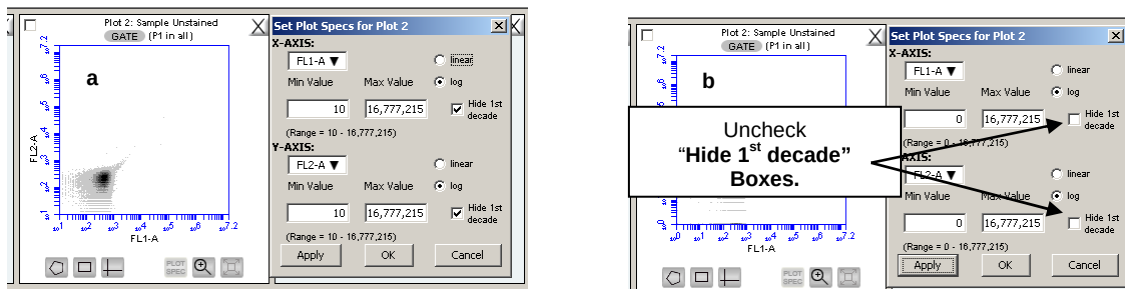


Figure 3-14 Selecting to display the 1st decade of data on a default log data plot. a. Open the Plot Spec dialog box. b. Uncheck “Hide 1st decade” boxes for FL1-A and FL2-A, and set the “Min Value” for both parameters to 0.

7. Add 4 more fluorescence density plots and apply the P1 gate to each. For this analysis, the plots shown in Figure 3-15 will be used to set appropriate fluorescence compensation.

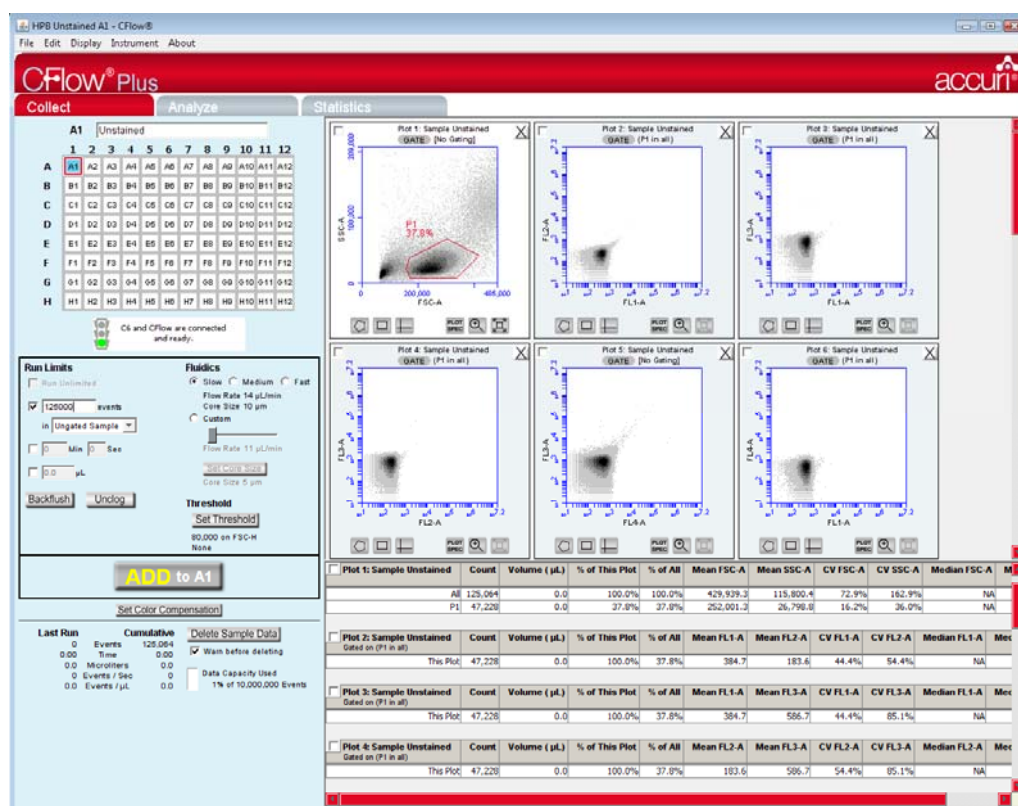


Figure 3-15 A CFlow Workspace with 5 added 2D fluorescence plots. Plots required for tutorial: FL1-A vs. FL2-A; FL1-A vs. FL3-A; FL2-A vs. FL3-A; FL4-A vs. FL3-A; FL1-A vs. FL4-A.

Adding Data to a CFlow File and Naming Plot Axes

1. Collect data from Sample 2, the CD45-PE-Cy7 tube. Sample 1 is removed from the SIP and replaced with Sample 2, after gentle re-suspension. There is no need to Backflush between samples. Residual sample in the SIP and flow cell is cleaned out automatically before the C6 begins data collection. You may wish to wipe the outside of the SIP with a lab wipe to minimize carryover between samples.
2. Click on an empty data well in the 96-well sample grid (well A2 is used in this example). The newly selected well is now outlined in red. The plots and gates created previously will remain, but will not contain data.

- Click the **RUN** button to start sample collection. During data collection data is displayed, and actively updated, in the plots. When the “run to” limit is reached the C6 stops sampling from the tube and well A2 turns blue to indicate that it now contains data (Figure 3-16).

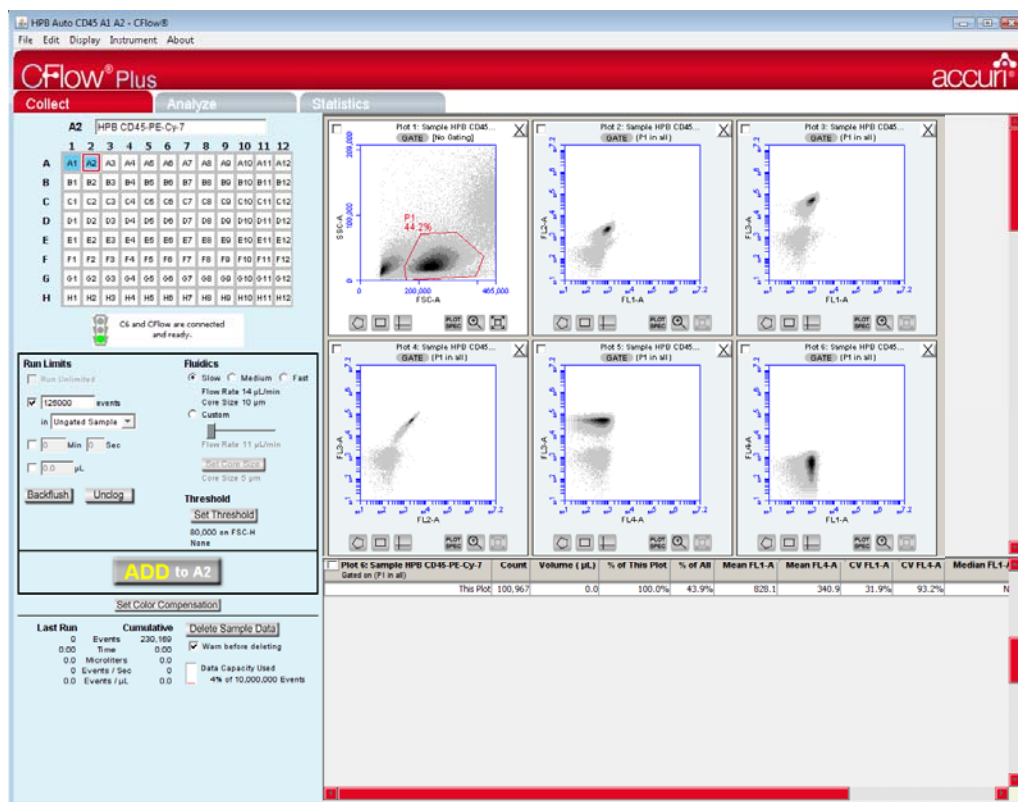
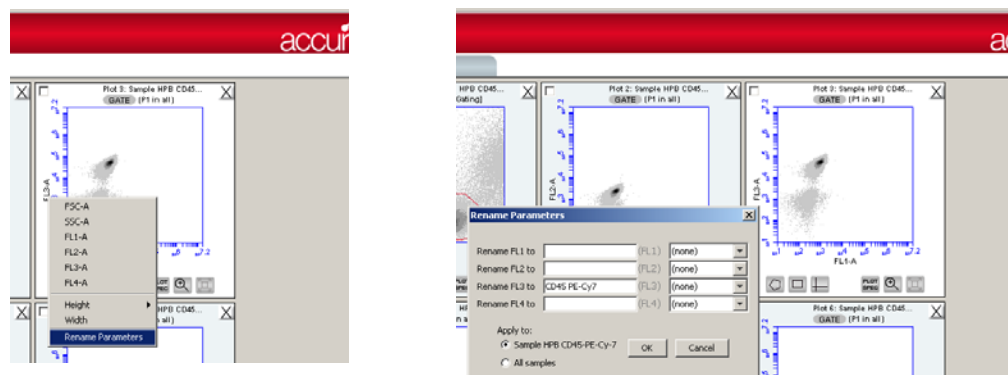


Figure 3-16 Sample number 2 has been added to well A2 in the CFlow Workspace.

- Change the axes labels to reflect the antibody staining or fluorochrome used. Clicking on any axis label will cause a menu to appear. Select the last option, **Rename Parameters** (Figure 3-17).



Click on axis label and select “Rename Parameters” from the drop down menu.

Enter axis label into the appropriate text box and click “OK”.

Figure 3-17 Changing axes labels using the axis label drop-down menu and the Rename Parameters option.

- Enter the label next to the parameter to be renamed in the **Rename Parameters** dialog box. In this example, CD45 PE-Cy7 is entered. The label can be applied to the current sample or to all samples by choosing the appropriate radio button at the bottom of the dialog box. Note that the label **CD45 PE-Cy7** will now become a selectable option to use for naming FL3 in other sample wells (Figure 3-

18).

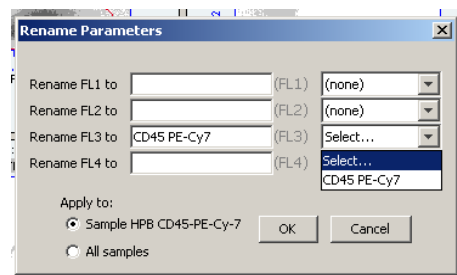
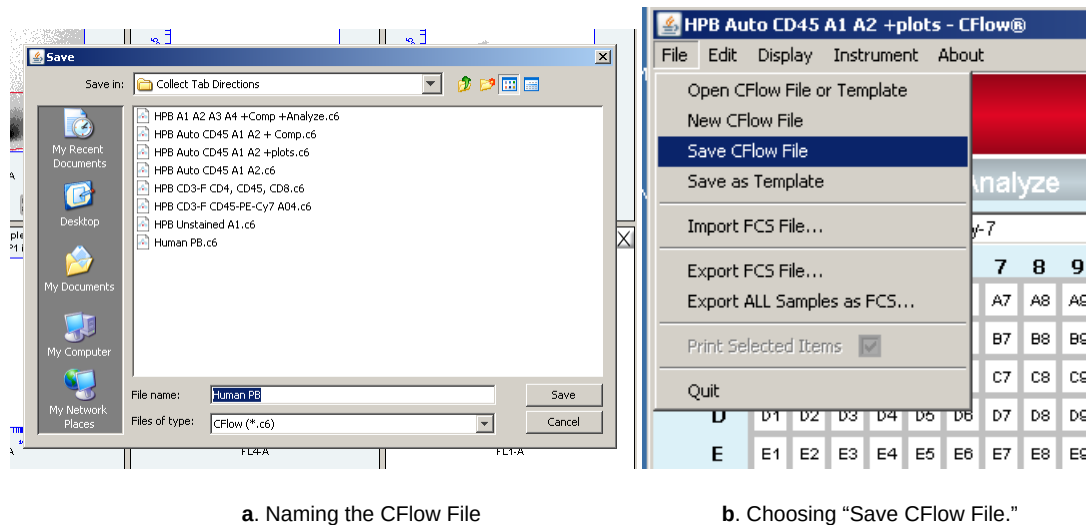


Figure 3-18 Changing axes labels using the axis label drop-down menu and the Rename Parameters option. Note that once the label CD45 PE-Cy7 has been entered it will now appear in the drop-down menu when the Select option is chosen.

Naming and Saving a CFlow File

Up to this point in the example, the data in wells A1 and A2 are saved only in the computer's RAM. The data will now be stored on the computer as a CFlow File.

1. Choose **File, Save CFlow File** from the drop-down menu in the CFlow command line (Figure 3-19b), enter an appropriate file name in the text box that opens (Figure 3-19a), and click **Save**. The file will automatically be given the extension .c6, indicating that the file has been saved as a CFlow data file.



a. Naming the CFlow File

b. Choosing "Save CFlow File."

Figure 3-19 Saving and naming a CFlow File.

Saving data as a CFlow file will save the CFlow workspace in its entirety, including sample data collected, along with plots, gating, compensation, and threshold settings. Not only will the **Collect** tab settings be saved, but so will any changes made in the **Analysis** or **Statistics** tabs. A CFlow file is a comprehensive (and often large) data file that saves instrument settings, FCS files, and plot layouts together. **Data collected on the C6 should always be saved as a CFlow file.**

CAUTION: CFlow does not automatically save CFlow files to the computer's hard drive. The data is only saved locally in RAM until the user saves the data as a CFlow file.

2. After the CFlow file name is applied, it will appear in the upper left corner of the CFlow file workspace (Figure 3-20).

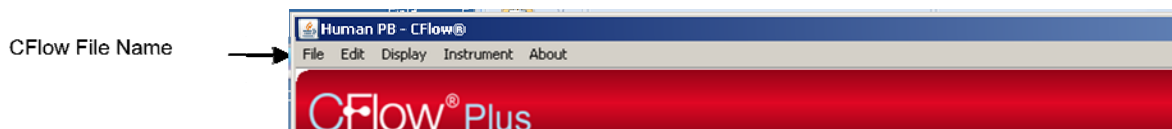


Figure 3-20 New file name, “Human PB”, appears in the upper left corner of the CFlow workspace after saving and naming the CFlow file.

- As more samples are run during this analysis session i.e., more data is added to this CFlow workspace, the CFlow File named “Human HPB” will need to be re-saved by selecting **File, Save CFlow File**, and overwriting the file. This should be done often to ensure against loss of data.

Calculating Median Values and Applying Fluorescence Compensation

A word about “fluorescence spillover”: In this example, some of the fluorescence signal from the CD45-PE-Cy7 positive cells is picked up not only in the FL3 channel, where it is expected, but also in the unstained FL1 and FL2 channels. This “spillover” is reflected in the shift to the right of the PE-Cy7 positive cells from the upper left into the upper right quadrants of each plot in Figure 3-21.

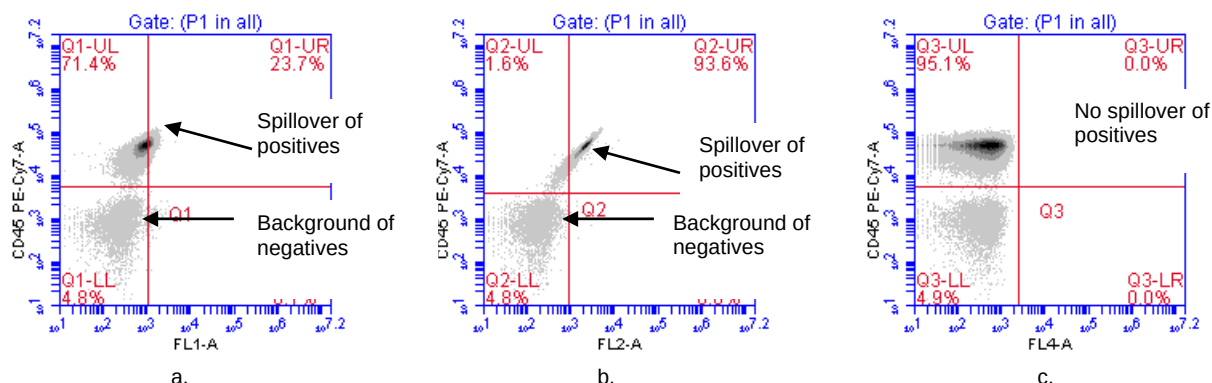


Figure 3-21 Fluorescence spillover of positive PE-Cy7 signal from FL3 into a. FL1-A, and b. FL2-A, c. The PE-Cy7 signal does not spillover into FL4-A.

This fluorescence spillover can be subtracted using a mathematical algorithm. This process is commonly referred to as “color compensation” or “fluorescence subtraction”.

- For each 2D fluorescence plot, choose the Quadrant Tool and click inside the plot to set the quadrant position (Figure 3-22).

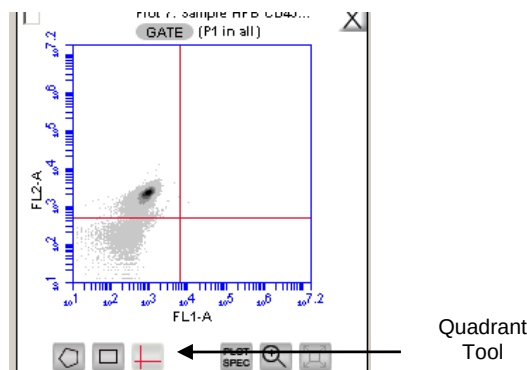


Figure 3-22 Selection and placement of Quadrant Marker

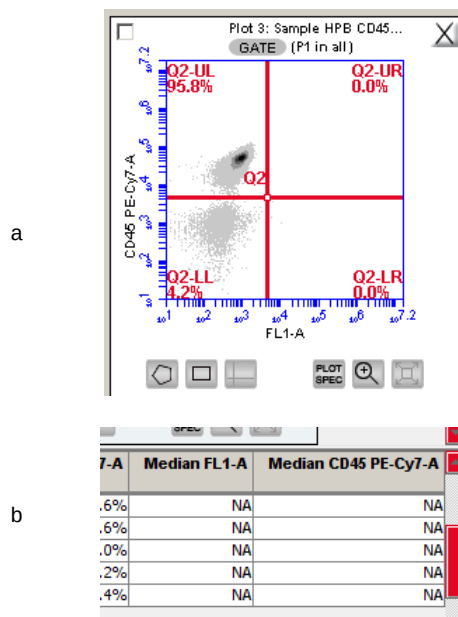


Figure 3-23 a. Placement of Quadrant Tool for Median calculations on the double negative (lower left) and single PE-Cy7 positive (upper left) populations. b. Median fluorescence values in the Statistics Table for Plot 3, Q1, will all read "NA" until Calculate Median is selected.

- On Plot 3 (FL1-A vs. CD45 PE-Cy7 plot), adjust the Quadrant Marker position as shown in Figure 3-23, so that the double negative and single PE-Cy7 positive populations are cleanly separated into the lower left and upper left quadrants, respectively.
- The Q2 quadrant marker and associated text in Plot 3 should appear bold, as in Figure 3-23. If not, click on any Q2 quadrant line to make it bold.
- Select **Display, Calculate Median** from the drop-down menu (Figure 3-24).

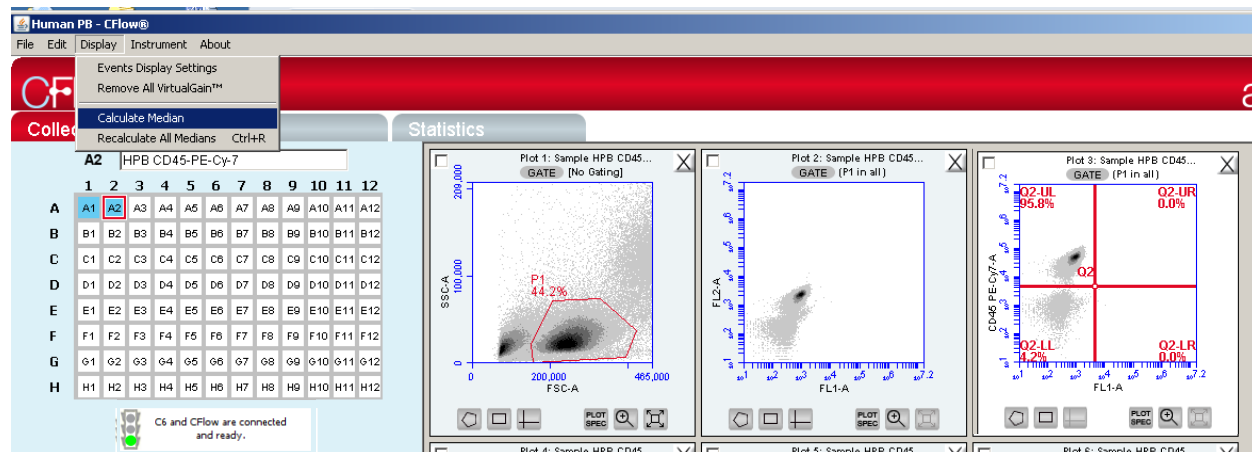


Figure 3-24 Quadrant Q2 is selected with mouse click (indicated by bold red) and Calculate Median chosen from the Display drop-down menu.

- The median fluorescence channel value for the events in each quadrant will now appear in the Statistics Table (Figure 3-25). However, no median is calculated for the top line, named **This Plot**. CFlow only calculates median values for events encompassed by Regions, Markers, Quadrants or Polygons.

Plot 3: Sample A2	Count	Median FL1-A	Median CD45 PE-Cy7-A
Gated on (P1 in all)			
This Plot	101,660	%	NA
Q1-UL	96,737	%	841.0
Q1-UR	0	%	0.0
Q1-LL	4,921	%	771.0
Q1-LR	2	%	1,322.0

Figure 3-25 Statistics Table updated with median values after choosing Calculate Median from the Display drop-down menu.

Color Compensation values to correct for fluorescence spillover will be calculated in this example using the median comparison method, outlined in the steps below. An alternative method is to utilize the C Comp Calculator Excel Spreadsheet provided by Accuri. Use of the C Comp Calculator saves time and improves accuracy when determining fluorescence subtraction values.

- Click on the **Set Color Compensation** button to open the **Compensation Settings** dialog box (Figure 3-26, lower left).

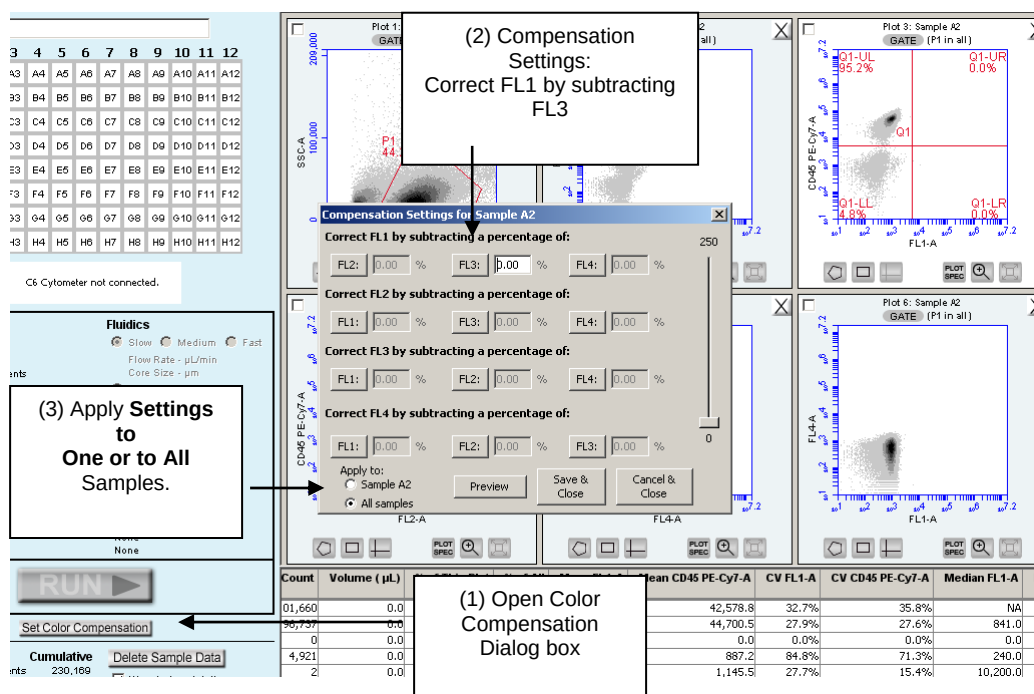


Figure 3-26 Opening and using the Compensation Settings dialog box.

- Click on the FL3 button in the middle of the top row, under the words **Correct FL1 by subtracting a percentage of:** in order to input a compensation value.

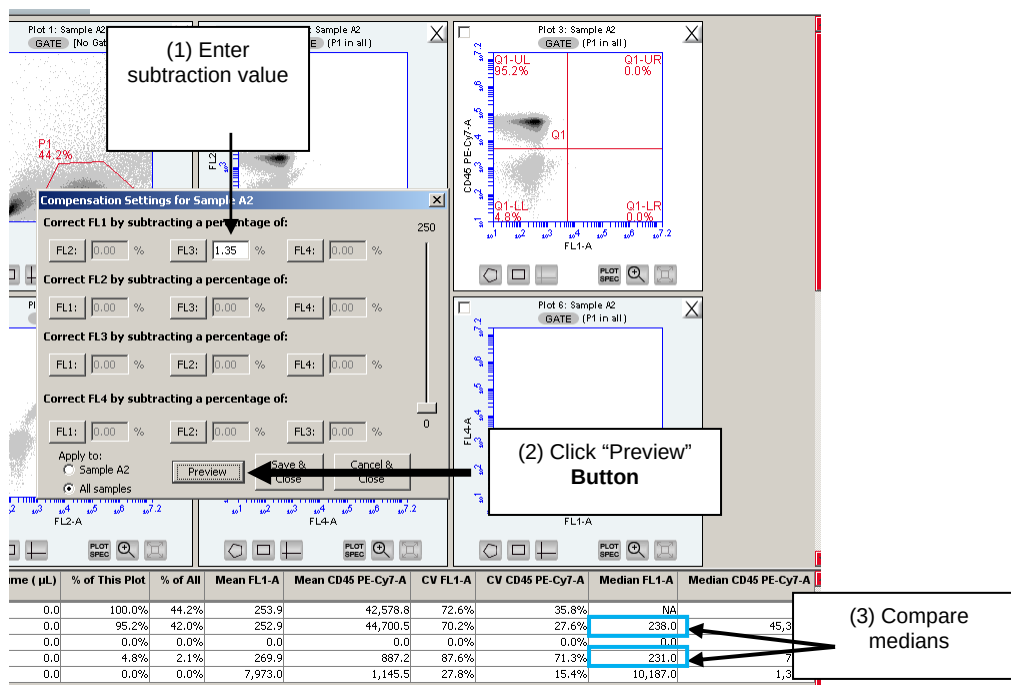


Figure 3-27 Results of subtracting spillover of FL3 PE-Cy7 signal from FL1 by 1.35%. Note that the median FL1 channel numbers in Q1-UL (238) and Q1-L (231) are approximately equal (blue boxes).

- Enter a subtraction percentage and click the **Preview** button in order to update the Statistics data. When the appropriate subtraction percentage is reached, the Median FL1 value in Q1-UL will be equal to that of FL1 in Q1-L, the negative population (Figure 3-27, values in blue boxes).
- Leaving the Compensation Settings dialog box open, click on Plot 4, FL2-A vs. CD45 PE-Cy-7, set Quadrants as in Figure 3-28, and select **Display, Calculate Median** from the drop-down menu.

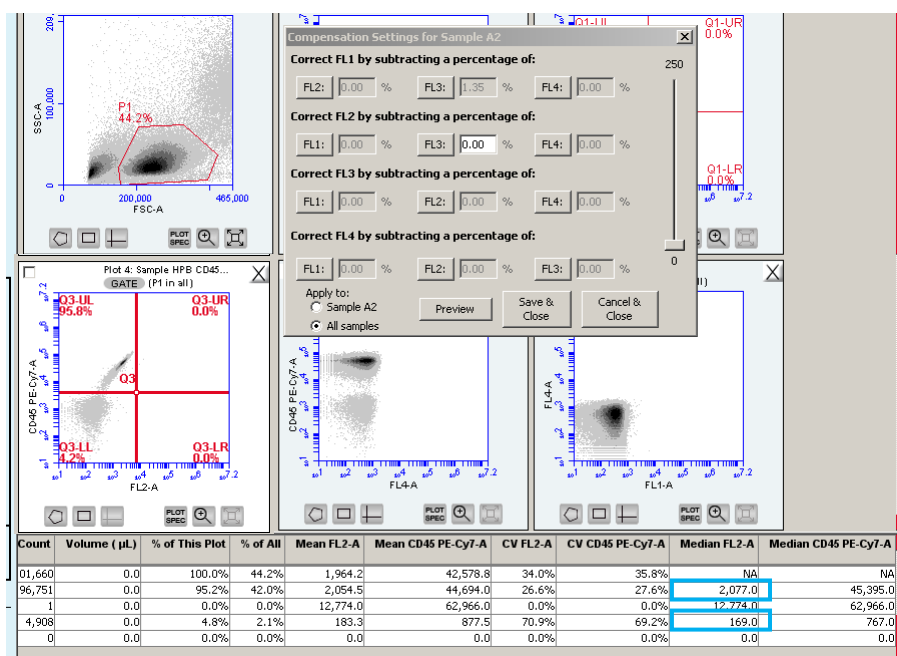


Figure 3-28 Results of selecting Plot 4, applying Quadrant markers and calculating Median fluorescence channel values.

10. Choose the “FL3” button in the second row of the Compensation Settings dialog box, under the line **Correct FL2 by subtracting a percentage of:** in order to input the appropriate subtraction value.
11. Enter a subtraction percentage and click the **Preview** button in order to update the Statistics data. For this example, the appropriate subtraction percentage is reached when the Median FL2 value in Q1-UL is equal to that of FL2 in Q1-LL, the negative population (Figure 3-29, blue boxed values).
12. Select **Apply to All samples**, then the **Save & Close** option in the Compensation Settings dialog box, in order to apply these Color Compensation settings.
13. The FL3 PE-Cy7 signal does not spillover into the FL4 channel significantly, as indicated by comparison of the Median FL4 fluorescence channel numbers (Figure 3-30). Therefore, there is no need to subtract FL3 signal from FL4.
14. The next sample collected is number 3, stained with CD45 PE-Cy7 and CD3 FITC (Table 3.1). There is significant spillover from the FL1 FITC signal into the FL2 channel (Figure 3-31a).

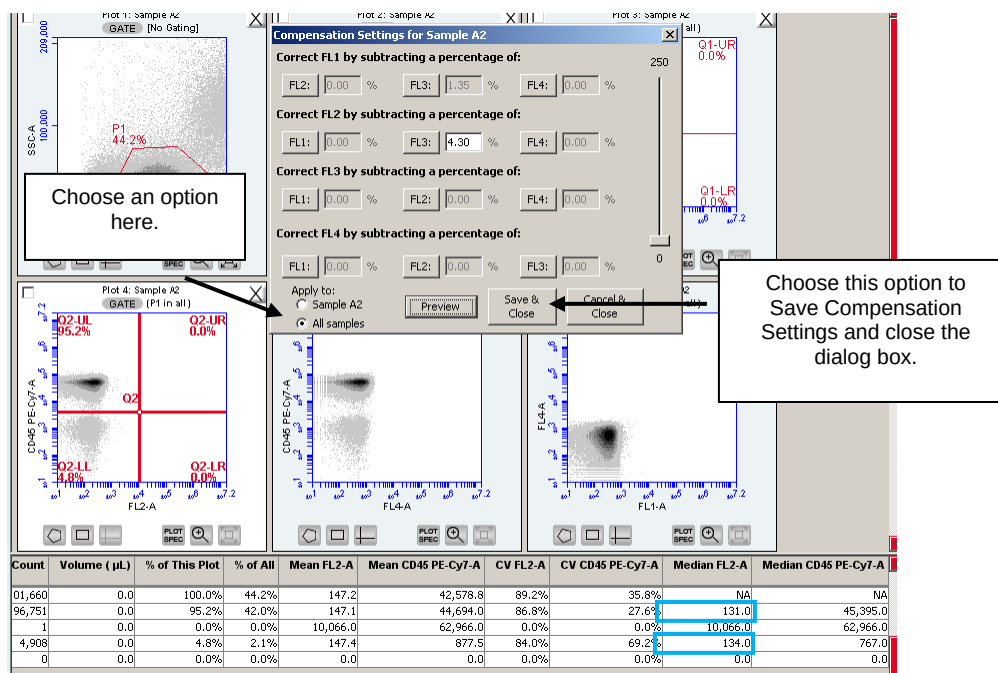


Figure 3-29 Results of correcting FL2 by subtracting 4.3% of the signal from FL3 (PE-Cy7). The Median FL2 fluorescence channel value for Q2-UL (131) is approximately equal to the Median FL2 fluorescence channel value for Q2-LL (134).

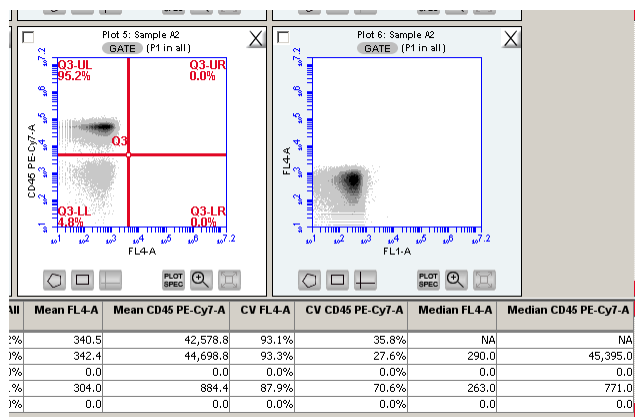


Figure 3-30 No fluorescence subtraction of FL3 PE-Cy7 signal is needed from FL4, as indicated by comparison of Median FL4 values for Q3-UL (290) and Q3-LL (263).

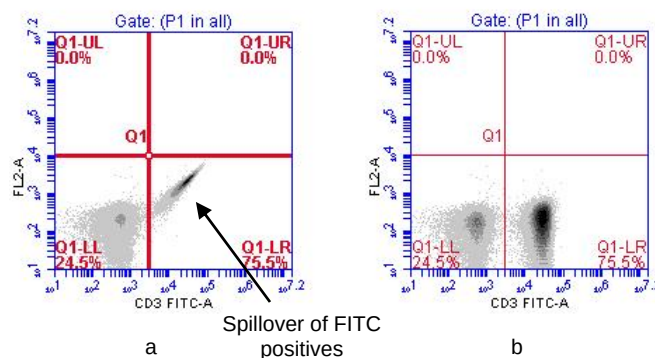


Figure 3-31 Correction of spillover from FITC FL1 into FL2. a. Before fluorescence subtraction. b. After calculation and application of appropriate percentage subtraction of FL1 from FL2.

15. Correct the spill of the FITC signal into FL2 by following steps analogous to 1 through 11 above, using Plot 2, with the following differences:

- Apply a Quadrant marker and calculate the Median values for Plot 2.
- In the Compensation Settings dialog box, enter a value in the FL1 box under the line “Correct FL2 by subtracting a percentage of:” Typically for any FITC conjugate, this value will be between 6.5 and 7.5 %.
- For this example, the correct compensation value will result in approximately equal median fluorescence channel values for FL2 events in the lower left and lower right quadrants of Plot 2.

Adding and Applying Additional Gates

In this example, CD45 will be used, in addition to the light scatter gate, to identify the CD3⁺CD4⁺ and CD3⁺CD8⁺ events. This section will show how to use the Gate dialog box to create this nested gate.

1. On Plot 3 (CD3-FITC vs. CD45-PE-Cy7), select the rectilinear gating tool and draw a region, R1, that encompasses the CD45 positive events (Figure 3-32). Be sure to slide R1 off the left edge of Plot 3 to ensure that all events, including those below channel 10 on the FITC axis, are in R1.

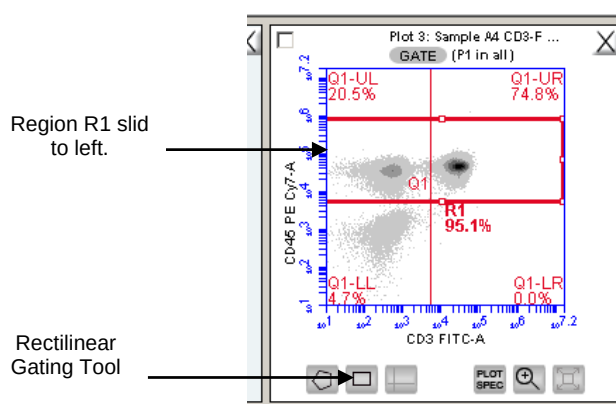


Figure 3-32 Gating region R1 has been drawn on Plot 3 to include the CD45-PE-Cy7 positive events.

2. Click the gray **Gate** button above Plot 2 (CD3-FITC vs. FL2-A) to open the Gate dialog box (Figure 3-33).
3. De-select the “Include” box next to the “P1 on All Events” option and select the “Include” box next to the option “R1 in (P1 in all)” (Figure 3-33).

4. Select the Apply button followed by the Close button at the bottom of the dialog box.
5. Repeat steps 1 to 4 above for Plot 6, CD3-FITC vs. FL4-A.
6. Move to well A4 and collect the data for Sample 4.
7. Save the changes to this CFlow file, by selecting **File, Save CFlow File**, and overwriting the current file.
8. See Figure 3-34 for a view of the Collect tab after application of the light scatter and CD45-PE-Cy7 positive gates to Plots 2 and 6, and collection of data for Sample 4.

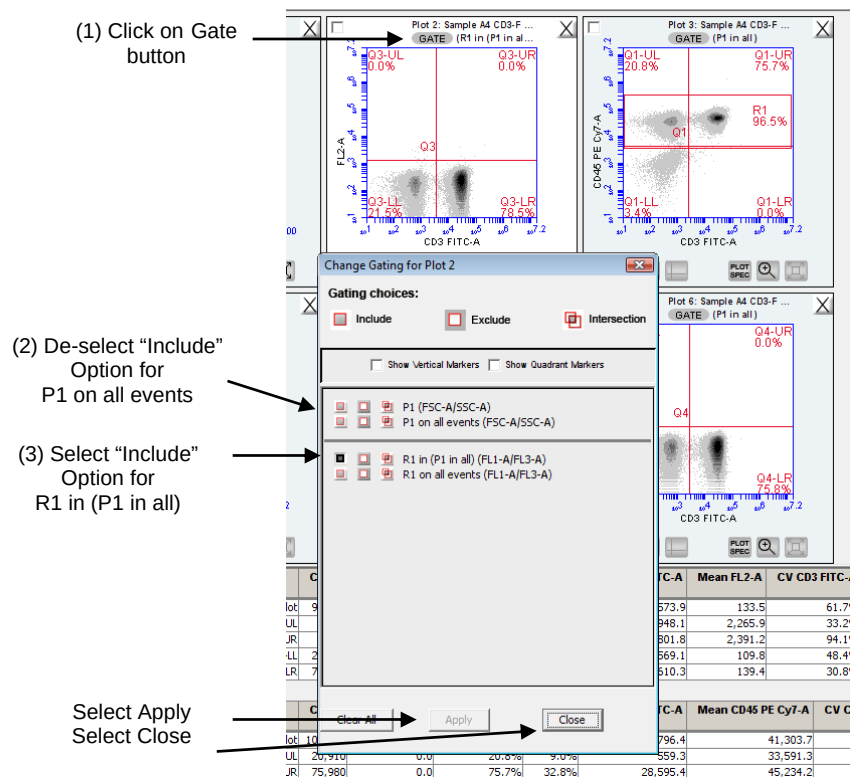


Figure 3-33 Gating Plot 2 (CD3-FITC vs. FL2-A) to include events in gates R1 and P1

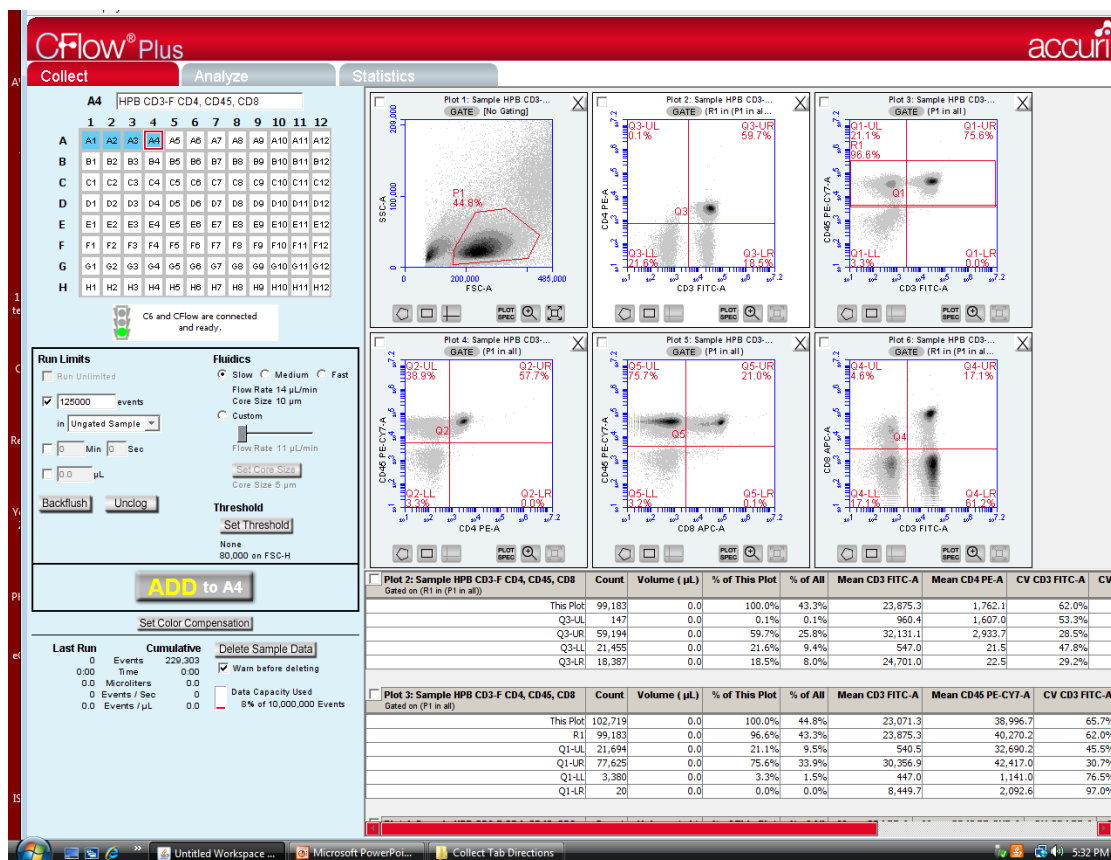


Figure 3-34 Gate for Plot 2 (CD3-FITC vs. FL2-A) includes events in gates R1 and P1, after following steps 1-3 in Adding and Applying Additional Gates.

Adjusting the Axes View Using the Plot Spec Tool (Zooming)

When CFlow plots are viewed with logarithmic scaling, data is displayed, by default, from channels 10 to 16.7×10^6 . For most analyses, only a small section of the axis needs to be viewed. In this section, the Plot Spec Tool will be used to select the desired channel range.

1. In order to better visualize the lymphocyte populations, the axes range for both Plot 2 (CD3 vs. CD4) and Plot 6 (CD3 vs. CD8) should be adjusted (Figure 3-35, blue boxes).

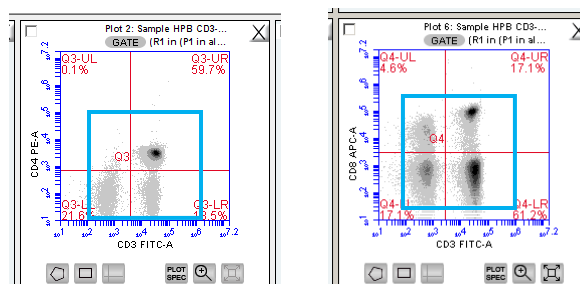


Figure 3-35 Blue boxes show desired 4 log channel to view for Plots 2 and 6.

2. Click on the Plot Spec Tool under Plot 2 (Figure 3-36).
3. Under **X-Axis**: set Min Value = 100 and Max Value = 1,000,000.
4. Under **Y-Axis**: set Min Value = 10 and Max Value = 100,000.

5. Select the **Apply** button to view the changes and then the **OK** button to close the Plot Spec dialog box.

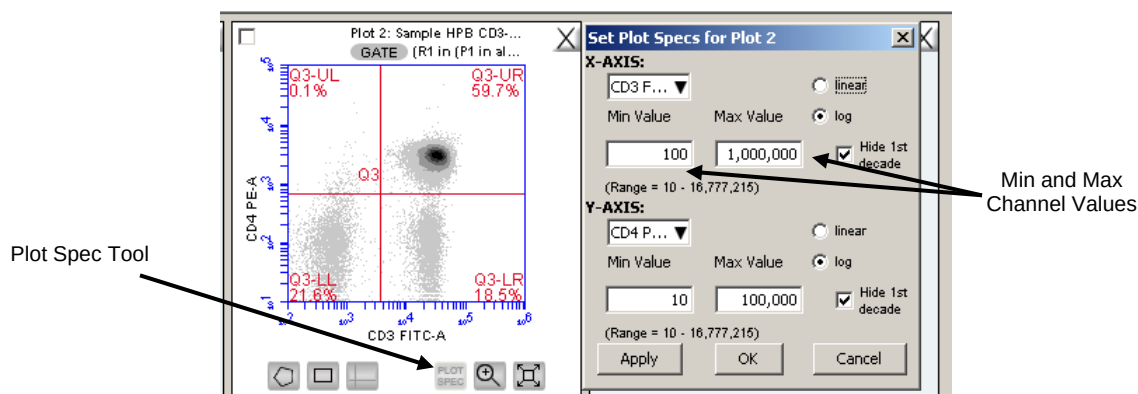


Figure 3-36 Setting Min and Max channel values for the X and Y axes using the Plot Spec dialog box.

6. Open the Plot Spec dialog box for Plot 6.
7. Under **X-Axis**: set Min Value = 100 and Max Value = 1,000,000.
8. Under **Y-Axis**: set Min Value = 50 and Max Value = 500,000.
9. Select the **Apply** button to view the changes and then the **OK** button to close the Plot Spec dialog box.
10. See Figure 3-37 for results of axes range adjustments.

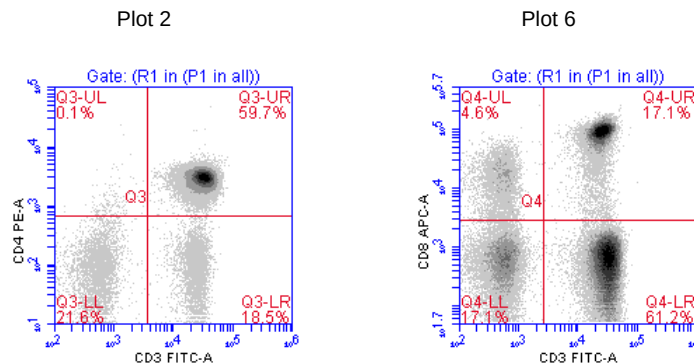


Figure 3-37 Result of axes view adjustment to 4 logs for Plots 2 and 6.

Chapter 4 Using the Analyze Tab

The Analyze tab is an easy way to view multiple data files simultaneously using the same plots and gating strategy. In this example of human peripheral blood analysis, the Analyze tab will be used to determine the percentage of CD45+CD3+ CD8+ and CD45+CD3+CD4+ cells.

1. Click on the Analyze tab near the top of the CFlow Workspace, just above the 96 well sample grid.
2. In the Analyze workspace plots can be copied from the Collect tab or new plots can be created (Figure 4-1). For this example, plots will first be copied from Collect.
3. Click the **Copy Plots from Collect** button and a dialog box will open (Figure 4-2).

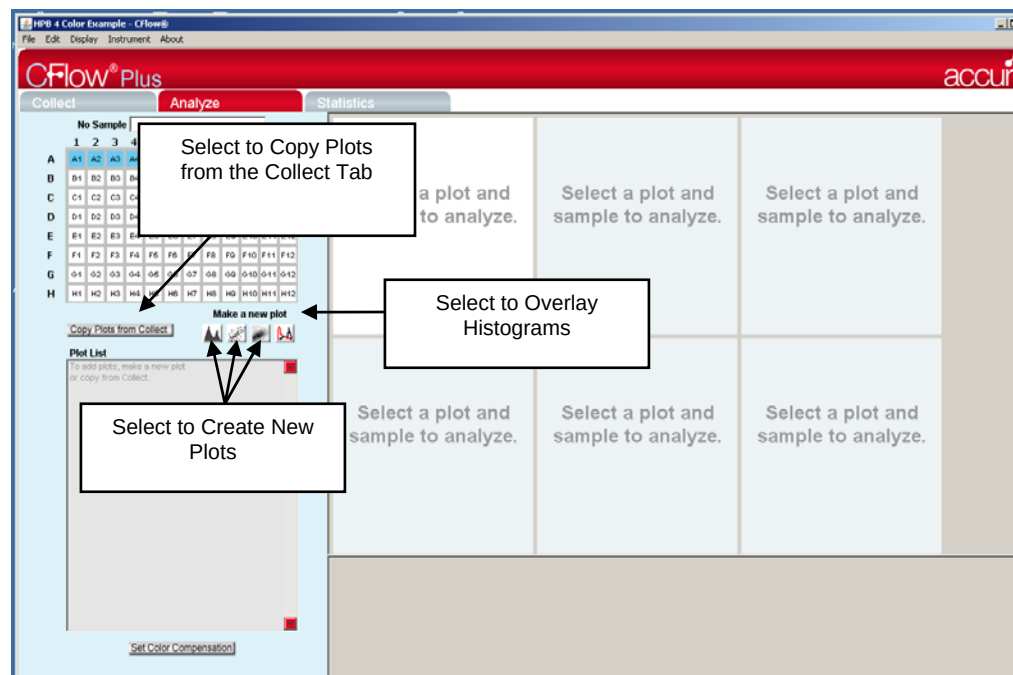


Figure 4-1 The Analyze Tab

4. Select the check boxes corresponding to the desired plots. For this example, choose Plot 1, Plot 2, Plot 3 and Plot 6 (Figure 4-2). Gating strategies applied in the Collect tab are not automatically applied in the Analyze tab.
5. Click OK. The dialogue box will close and the selected plots will be added to the Plot List (Figure 4-3).
5. Click on the upper left plot corral in the Analyze tab to select it (background turns from blue to white), and then select Plot 1C (FSC-A/SSC-A) from the Plot List on the left hand side of the page (Figure 4-3).

Note that the gate around the lymphocyte population is now named “P2” (Figure 4-4). “P2” is a copy of “P1” from the Collect tab. “P2” can now be adjusted in the Analyze tab without changing the position of “P1” in Collect tab.

6. One by one, select the next 2 empty plot corrals in the top row and choose Plot 3C (FL1-A/FL3-A) and Plot 6C (FL1-A/FL4-A), respectively, from the Plot List (Figure 4-4).
7. Display the data for Sample well A1 in each of these plots by clicking on the plot, then clicking on well A1 (Figure 4-4).
8. On Plot 3C, click on the Gate button and select to include “P2 on all events”.
9. On Plot 6C, click on the Gate button and select to include “R2 in (P2 in all)” (Figure 4-5).

10. In the second row of plot corrals, open Plots 1C, 3C and 6C, this time choosing Sample A2 data to display in each plot (Figure 4-6). The gates set on Plots 3C and 6C in Steps 8 and 9 are applied automatically to the appropriate plots in Row 2.

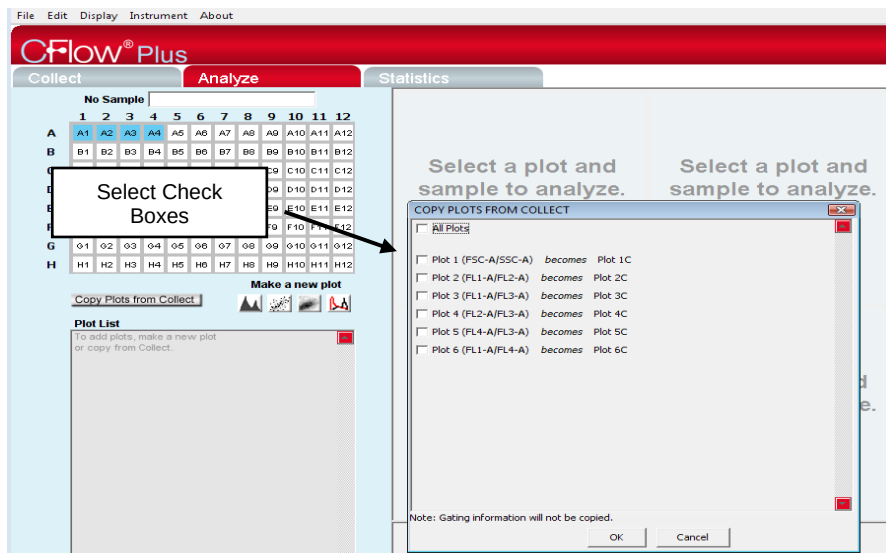


Figure 4-2 Selecting plots to copy from the Collect tab.

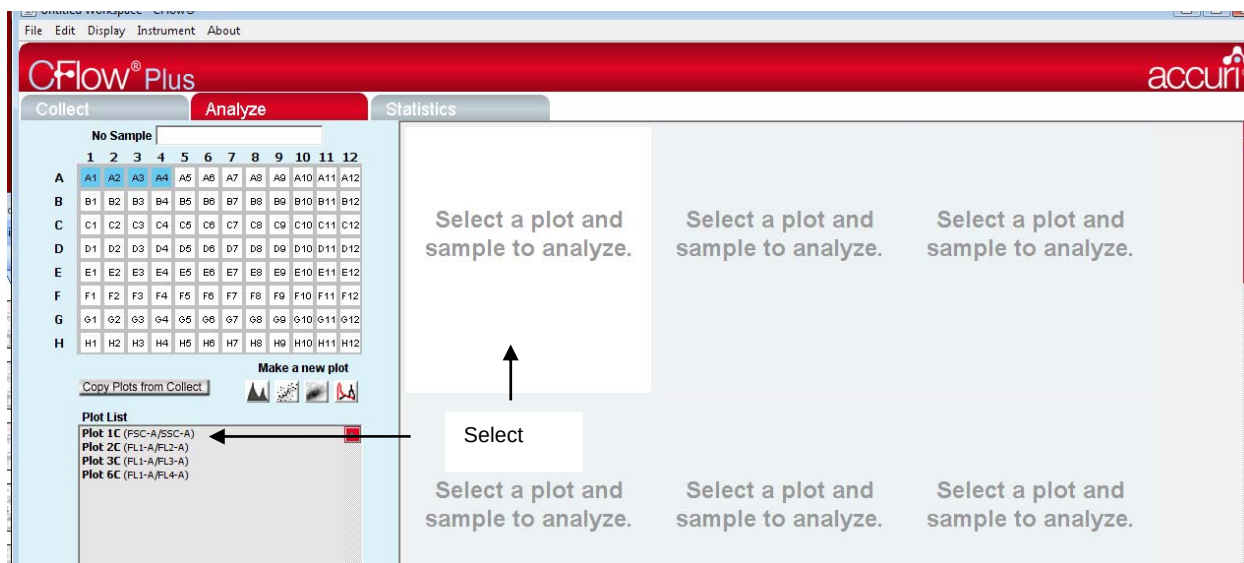


Figure 4-3 Plots 1C, 3C and 6C opened in the top row of Analyze tab

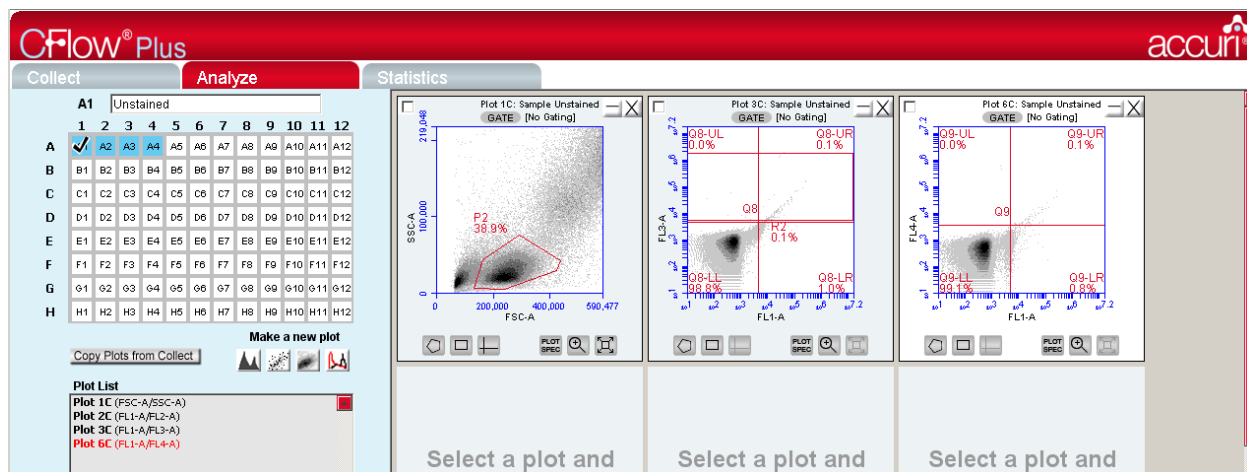


Figure 4-4 Analyze tab with copied plots in Plot List and selected plot corral.

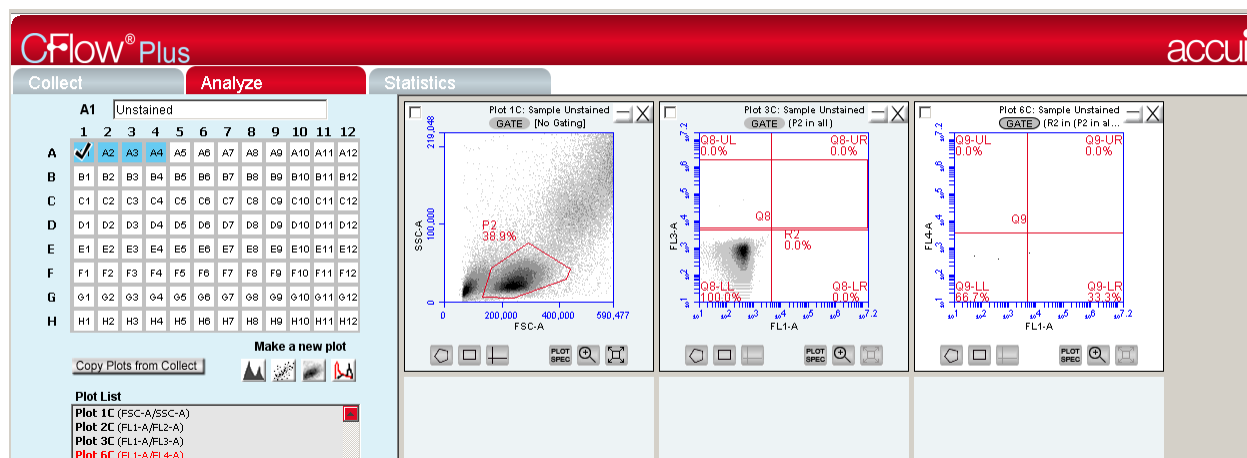


Figure 4-5 Analyze Tab: Result of gating Plot 3C on "P2 in all" and Plot 6C on "R2 in (P2 in all)"

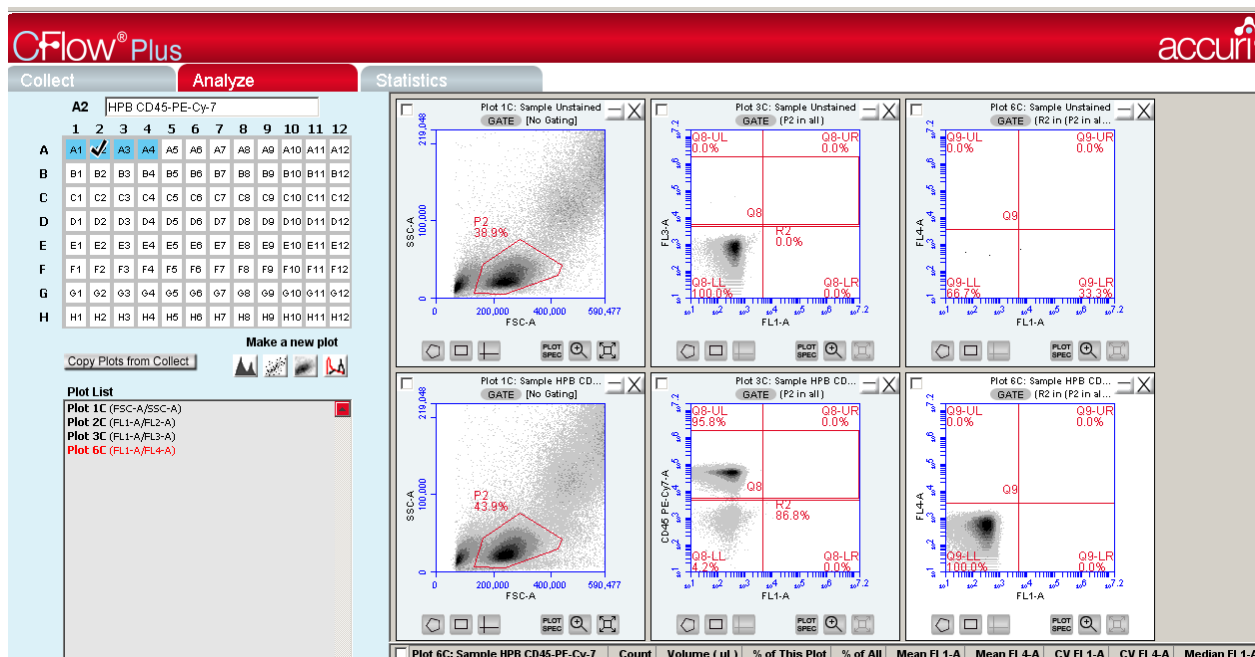


Figure 4-6 Analyze Tab: Row 2 selected to display data from Sample A2 in Plots 1C, 3C and 6C.

11. Use the Scroll Bar on the right hand side of the Analyze tab Workspace to scroll down, bringing six more plot corrals into view (Figure 4-7).

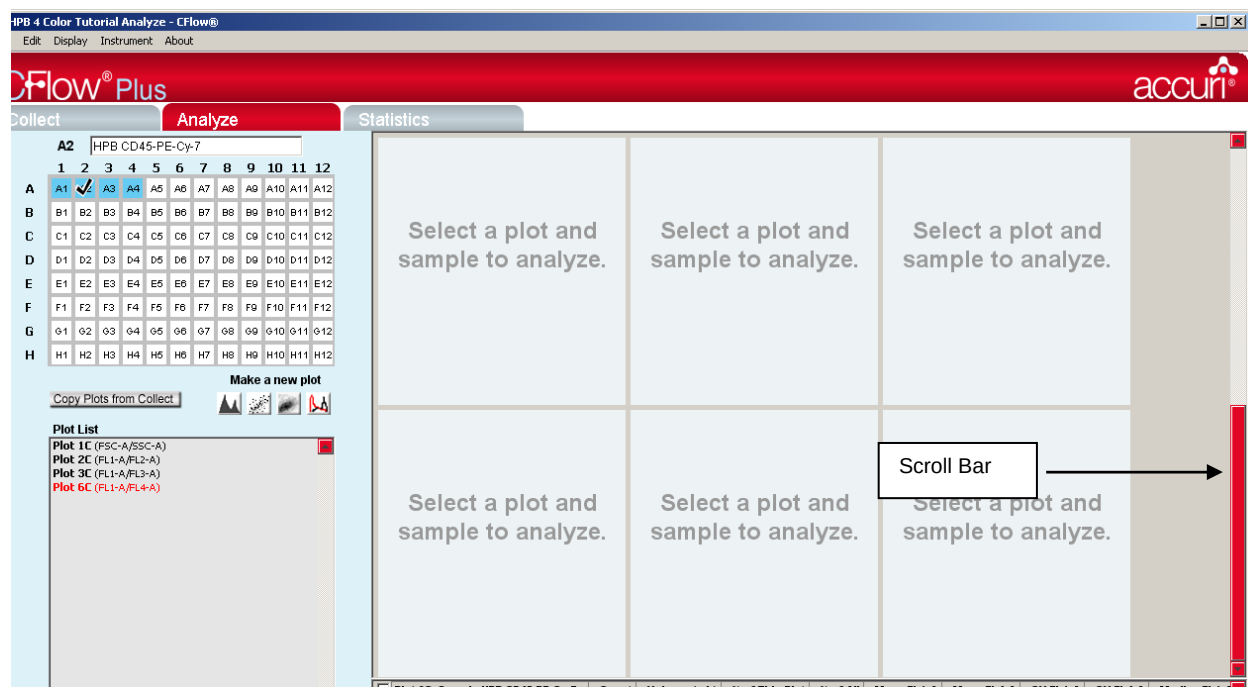


Figure 4-7 Analyze Tab view after scrolling down to empty plot corrals.

12. Select Plots 1C, 3C and 6C for these two rows of the Analyze tab, and populate the plots with data from Sample A3 (in Row 3) and Sample A4 (in Row 4). (Figure 4-8).

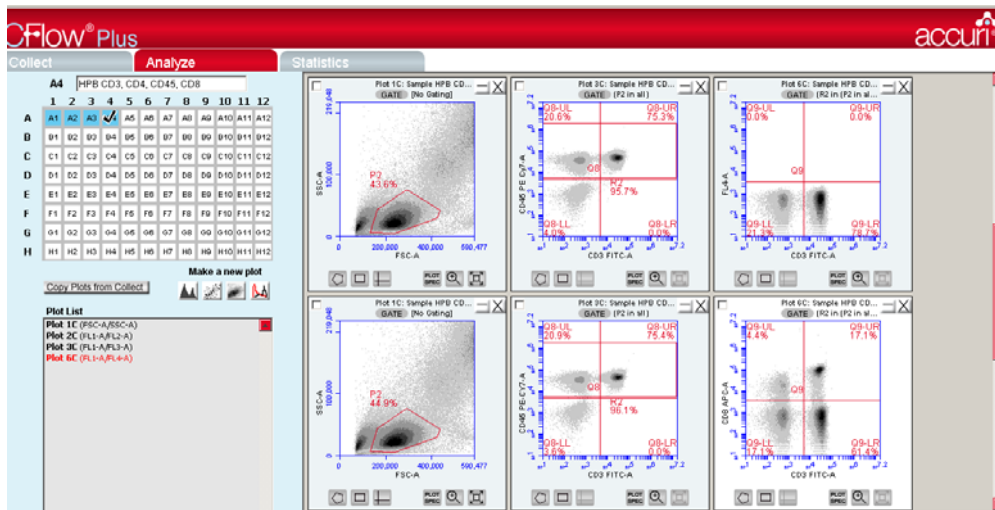


Figure 4-8 Analyze Tab view after adding 2 more rows of Plots 1C, 3C and 6C, and populating with data from Sample A3 and A4.

- To print directly from the Analyze tab, click on the small white box in the upper left corner of each plot you want to print, and then select **File, Print Selected Items** from the drop-down menu. The corresponding Statistics table for each plot will automatically be selected as well. These can be de-selected with a click, if desired (Figure 4-9).

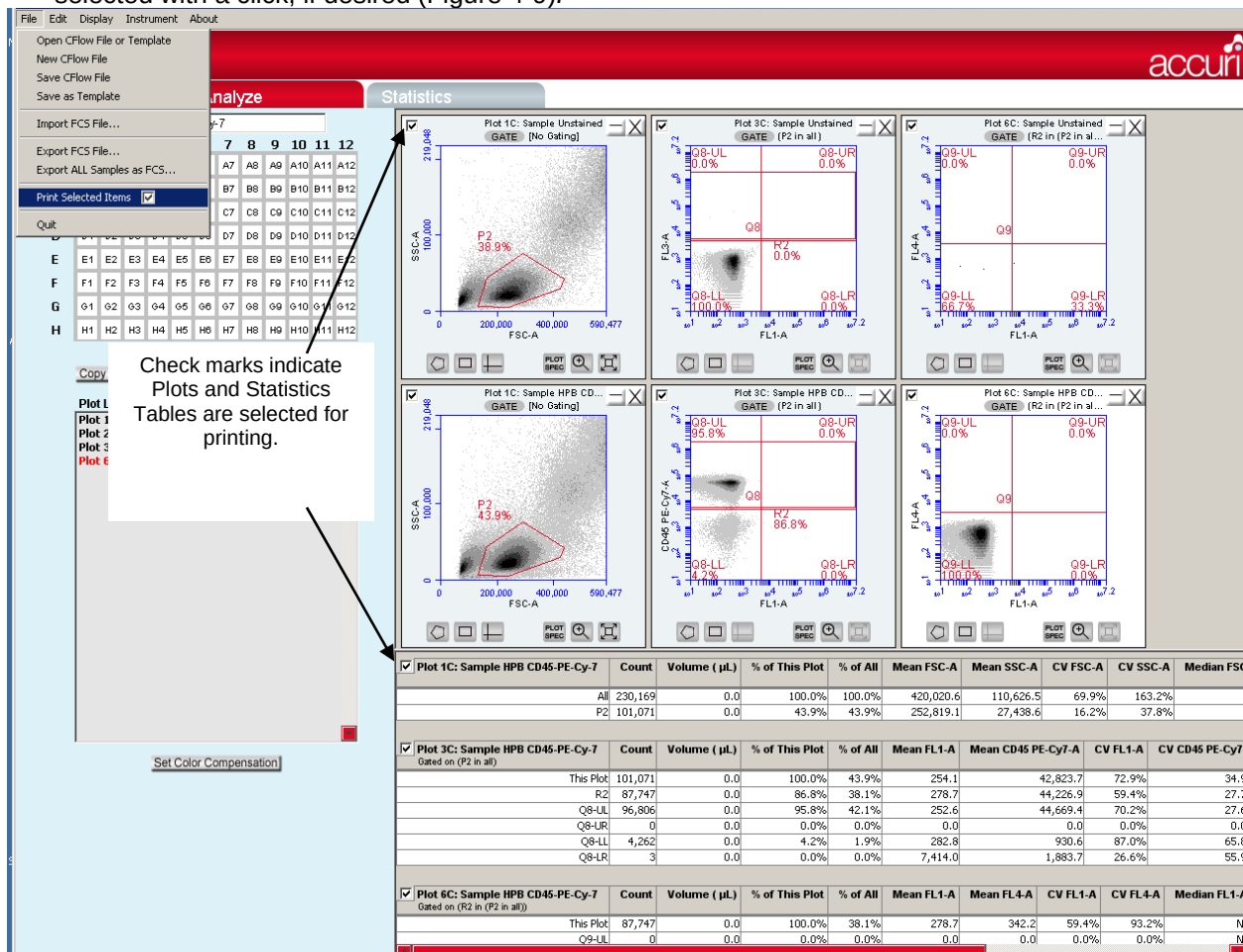


Figure 4-9 Analyze Tab: Plots are selected for printing.

Make an Overlay Histogram in Analyze Tab

1. Scroll down to an empty plot corral and select it (background will turn from blue to white).
2. Click on the Overlay Tool, under the 96 well grid, and a single parameter, FSC-A plot will open (Figure 4-10).
3. Click on the axis label (FSC-A) and change to the desired parameter. Use FL1-A if working with the HPB 4 Color Tutorial file.
4. Click on the Gate button and apply the desired gate (R1 in (P1 in all) for the HPB 4 Color file).
5. Select the data wells from the 96-well grid to be overlaid. Choose samples A2, A3 and A4 if using the HPB 4 Color file (Figure 4-11).

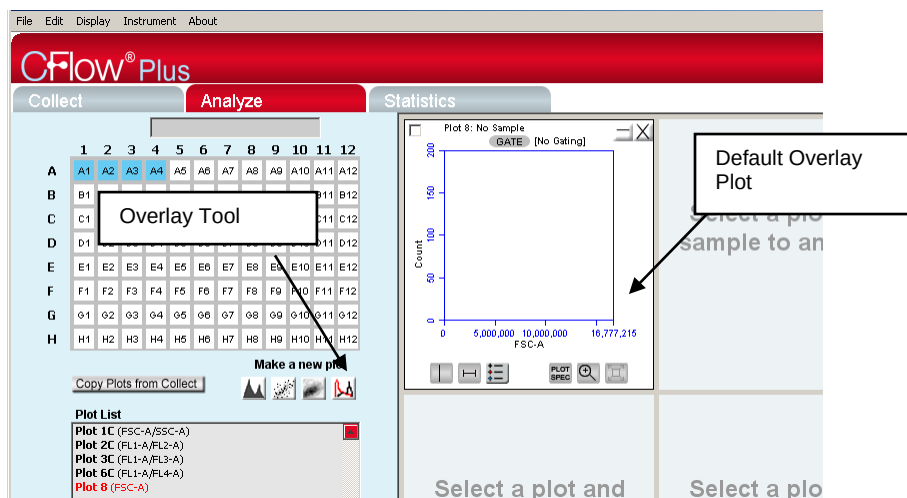


Figure 4-10 Analyze tab: Overlay Tool has been selected.

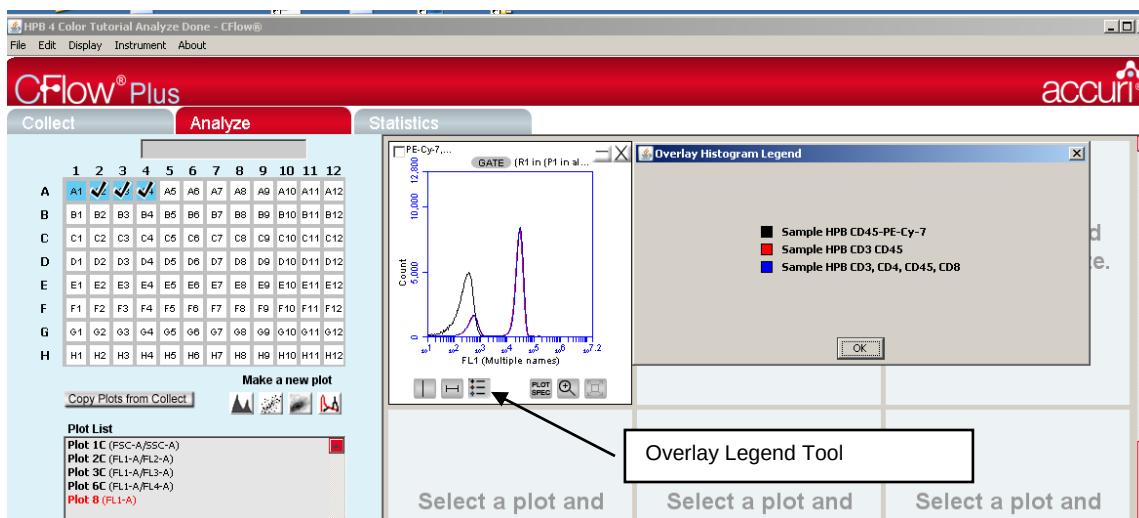


Figure 4-11 Analyze tab: Overlays of FL1-A data for samples A2, A3 and A4. The Overlay Legend has been opened.

6. Selecting the Overlay Histogram Legend Tool opens the dialog box shown in Figure 4-11.

All plots created in the Analyze and Collect tabs, including Overlays, can be dragged and dropped into most Microsoft Office compatible programs. Statistics Tables can be copied and pasted, as well.

Chapter 5 CFlow Software Menus

The File Menu

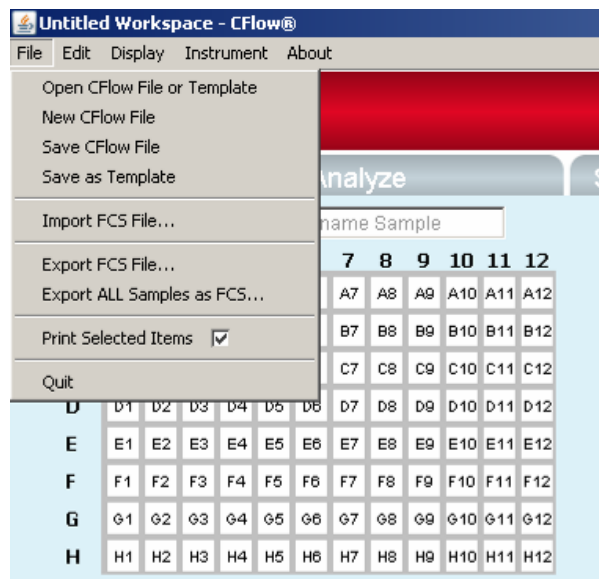


Figure 5-1 The File Menu

Open CFlow File or Template: Opens a pre-existing CFlow file or CFlow Template. Only one CFlow workspace can be opened at a time.

New CFlow File: Opens a new, blank CFlow workspace, replacing the existing open workspace.

Save CFlow File: Allows re-naming and saving of the currently open CFlow workspace.

Save as Template: Creates a template from the currently open CFlow workspace. All markers, regions, gating, parameter names, sample names, etc. will be saved without any data points.

Import FCS File...: Allows importation of a FCS file previously exported from another CFlow file to the currently open workspace. Only FCS files that were created using a C6 can be imported into CFlow.

Export FCS File...: Exports and saves the currently selected data well (sample) as a FCS 3.0 file to a user- selected folder.

Export ALL Samples as FCS...: Saves all the data wells (samples) as individual FCS 3.0 files in the folder "CFlow-FCS Exports" on the desktop.

Print Selected Items: Prints the selected plots and associated statistics. At least one plot should be checked in the **Print** check box, located in the upper left hand corner of each plot, for this option to be enabled. The Statistics Box associated with each selected plot will automatically be selected for printing. The selected plots will print Landscape orientation on the first page with the associated statistics in Landscape orientation on the second page. The **Print** check box can be unchecked for any given Statistics Box in order to print plots alone.

Quit: Quits CFlow and closes the application. CFlow will always prompt to Save the Workspace upon selecting this option.

The Edit Menu

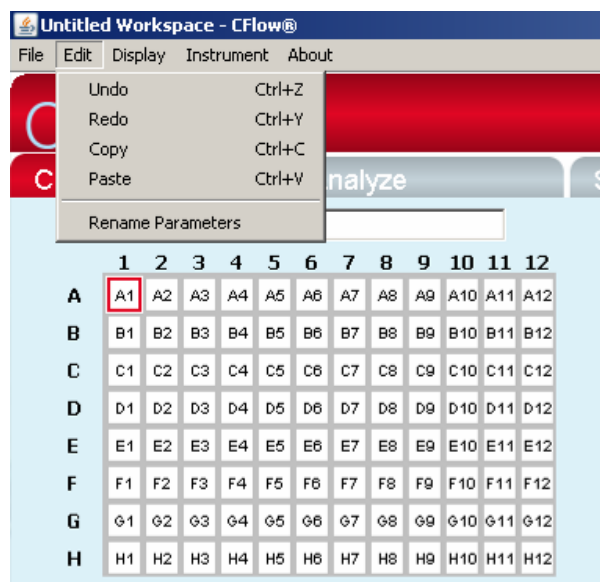


Figure 5-2 The Edit Menu

Undo: Undoes the last action that was performed in CFlow. Shortcut = **Ctrl+Z**. Not all actions are undoable. For example, deleting data using the **Delete Sample Data** button cannot be undone using this command.

Redo: Redoes the last action done. Shortcut = **Ctrl+Y**. See **Undo**.

Copy: Allows for a copy of a marker or region to be pasted into another plot. It also allows for copying of statistics from the tables in CFlow to other programs such as Microsoft Office. Shortcut = **Ctrl+C**. To Copy and Paste plots from a CFlow workspace, left click anywhere on a plot and drag it to an open application. Ctrl+C cannot be used in conjunction with Ctrl+V to copy and paste plots from CFlow into other applications.

Paste: Allows for the pasting of previously copied markers and regions into new plots. Shortcut = **Ctrl+V**. Ctrl+V cannot be used in conjunction with Ctrl+C to copy and paste plots into other applications.

Rename Parameters: Allows renaming of individual parameters in either the current sample or all samples at the same time. The dialogue box shown in Figure 5-3 will appear when this option is selected. This option can also be accessed by clicking on a parameter label within a plot.

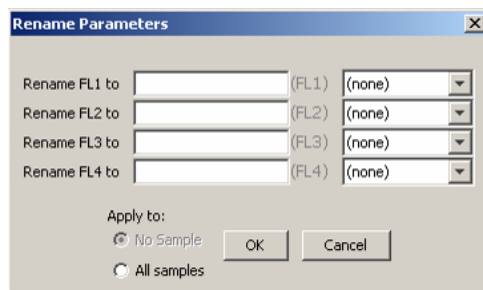


Figure 5-3 Rename Parameters

Once a parameter has been renamed, that name will appear as an option in the drop-down menu under the word "Select" (right hand column of the Rename Parameters dialog box, Figure 5-4).

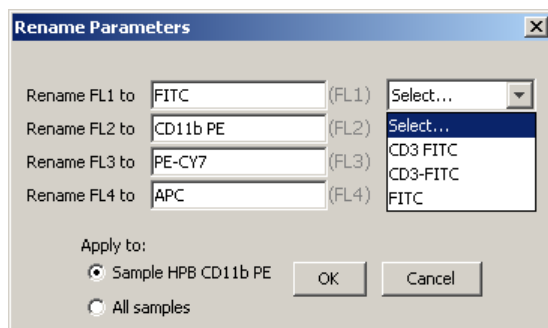


Figure 5-4 Rename Parameters, selection options menu

The Display Menu

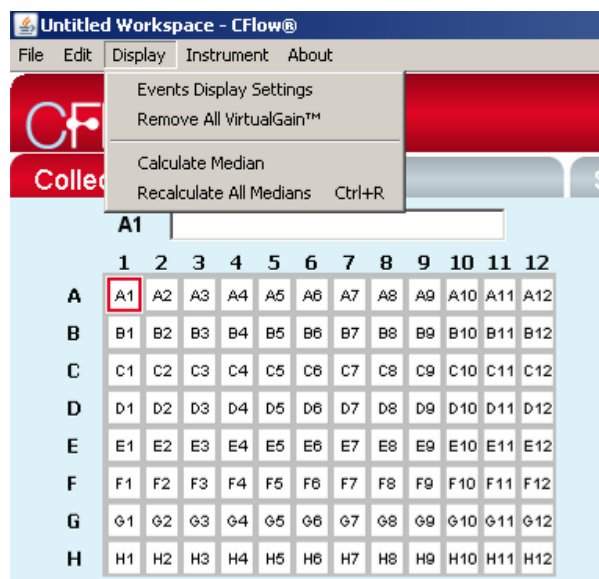


Figure 5-5 The Display Menu

Events Display Settings: Used to change the number of events displayed in all plots. There are 3 options: display all events acquired, display the first specified number of events in the data file, or display a percentage of the whole. If percentage is chosen, it will be a pseudo-random selection. For example, if 20% is selected, every 5th event will be displayed.

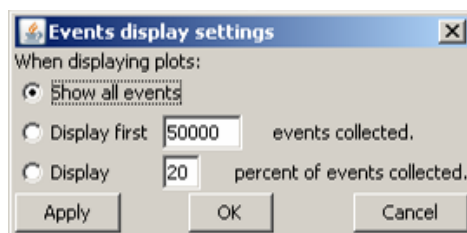
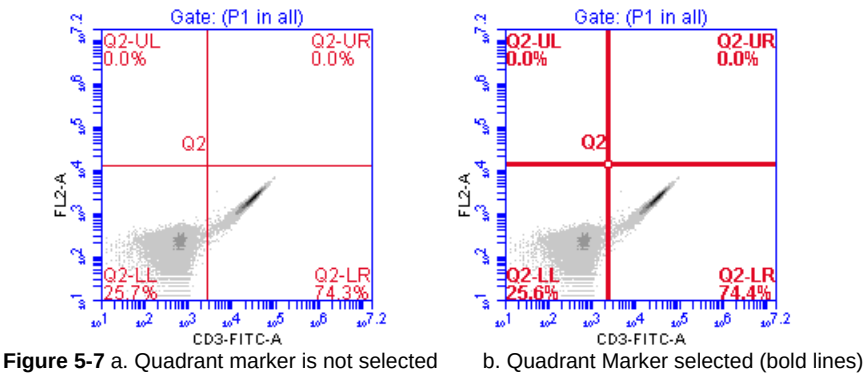


Figure 5-6 Events Display Settings

Remove All VirtualGain™: Removes all VirtualGain settings that have been applied from the entire CFlow workspace. VirtualGain settings will be removed from all wells of data. Refer to the VirtualGain chapter for more information.

Calculate Median: Before selecting this option, the marker or region where the median is required should be made bold by clicking on it with the mouse (Figure 5-7b). If a median has never been calculated for a marker or region the notation “NA” will appear in the Statistics Table (Figure 5-8a). Once the “Calculate Median” option has been selected, the result will be displayed in the statistics pane of the Collect or Analyze tabs (Figure 5-8b).



FL2-A	Median CD3-FITC-A	Median FL2-A
1.4%	NA	NA
3.0%	NA	NA
3.0%	NA	NA
1.0%	NA	NA
3.0%	NA	NA

FL2-A	Median CD3-FITC-A	Median FL2-A
4%	NA	NA
0%	0.0	0.0
0%	0.0	0.0
0%	560.0	193.0
1%	29,490.0	2,121.0

Figure 5-8 a. Statistics table before selecting Calculate Median b. Statistics table after selecting Calculate Median

Recalculate All Medians: If a marker or region for which a Median value has been calculated is moved, the Median will not automatically recalculate. The notation “tbd” (to be determined) will appear in the Statistics Table (Figure 5-9). **Recalculate All Medians** should be selected to update the value (Figure 5-8b). The first statistics line will always read NA. There is no calculation of the All row.

FL2-A	Median CD3-FITC-A	Median FL2-A
4%	NA	NA
0%	tbd	tbd
0%	tbd	tbd
1%	tbd	tbd
0%	tbd	tbd

Figure 5-9 a. Statistics table after moving Quadrant markers

The Instrument Menu

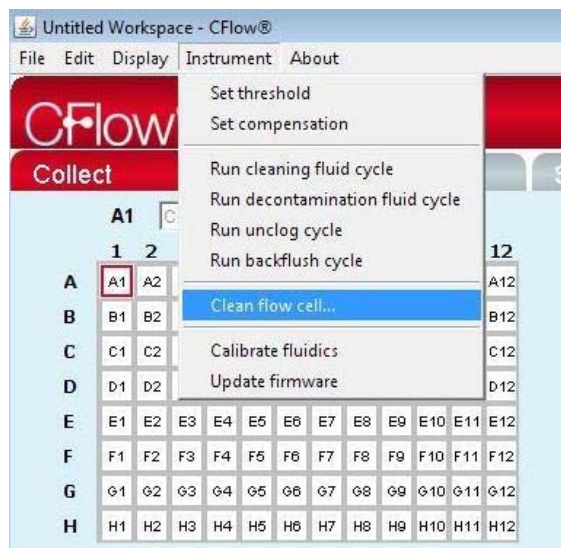


Figure 5-10 The Instrument Menu

Set Threshold: Opens the Threshold dialogue box (Figure 5-11). This allows for selecting the trigger channel, setting the Primary Threshold value, and setting an **optional** Secondary Threshold. Changing the Primary Threshold to a parameter other than FSC-H will change the parameter that is being used to trigger the data collection. The threshold settings can be applied to either the sample currently being viewed or all the samples. Selecting **All Samples** will apply these settings to all of the previously collected data in the open CFLOW workspace. This command is also available directly from the Control Panel via the **Threshold** button.

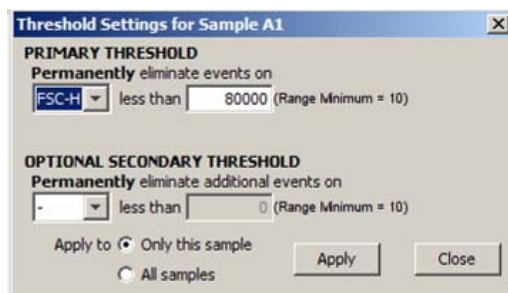


Figure 5-11 Threshold Settings

Take care when setting the Primary and Secondary Thresholds. When the thresholds are set before or during data collection, any events not meeting the threshold criteria will not be acquired or saved. When changes are made to the threshold values after data collection, a warning message will appear if changing the threshold value will result in permanent loss of data not meeting the new threshold criteria from the data file (Figure 5-12).

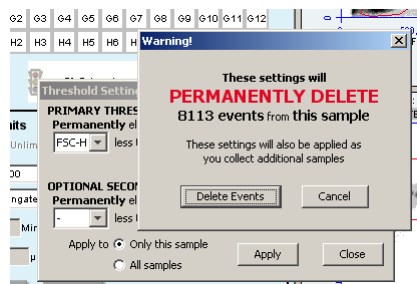


Figure 5-12 Warning displayed after a change in threshold settings

Set Compensation: Opens the **Compensation Settings** dialogue box. The Compensation Settings dialog box can also be accessed from the instrument settings panel of the Collect tab, under the **Run** button.

Click on the parameter to be subtracted in order to enter a percentage subtraction value. In the example shown in Figure 5-13, the **FL2** button in the upper left corner has been clicked and the numbers in the percent box appear on a white background. A subtraction percentage value may now be entered. A grayed out box is not accessible until the parameter button to its left is clicked.

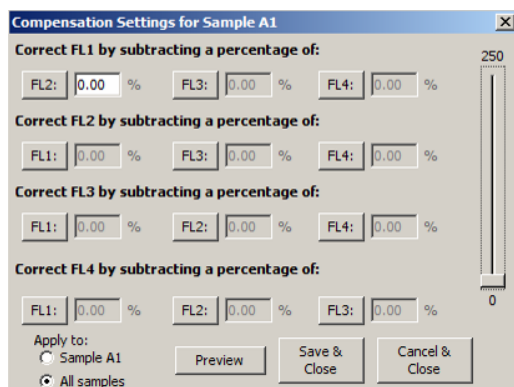


Figure 5-13 Compensations Settings

Compensation can be applied to the currently viewed sample or all samples simultaneously by choosing one of the options under the words **Apply to** in the lower left hand corner.

The Compensation dialog box can remain open and in view throughout data collection and analysis – there is no need to close it when moving between samples, making or changing plots and applying gates, or even when moving between the Collect, Analyze and Statistics tabs.

Run Cleaning Fluid Cycle: Runs a cleaning fluid cycle. This cycle takes ~16 minutes to complete and is different than the **Decontamination Fluid Cycle** that occurs during shut down.

Run Decontamination Fluid Cycle: Manually runs the **Decontamination Fluid Cycle** that is performed automatically during a normal shut down of the C6.

Run Unclog Cycle: Runs the Unclog cycle. This command is also directly available from the Control Panel via the **Unclog** button. Running the Unclog Cycle will cause fluid to drip out of the SIP. Be sure to remove your sample tube and place a blotter or empty sample tube under the SIP to catch the effluent.

Run Backflush Cycle: Runs the Backflush cycle. This command is also directly available from the Control Panel via the **Backflush** button. Running the Backflush Cycle will cause fluid to drip out of the SIP. Be sure to remove your sample tube and place a blotter or empty sample tube under the SIP to catch the effluent.

Clean Flow Cell: The flow cell will fill completely with cleaning solution aspirated from the sample tube on the SIP. This cycle will automatically shut down the C6 with cleaning reagent that will remain in the flow

cell, allowing the flow cell to “soak”. When the C6 is started up again after this cycle, a longer fluidics Startup cycle will run and CFlow will indicate that an “improper shut down” has occurred. This longer cycle will purge cleaning reagent from the flow cell and the C6 will be ready to use when Startup is complete.

CAUTION: Never run the Clean Flow Cell cycle without a tube containing at least 250 µL of fluid on the SIP.

Calibrate Fluidics: Initiates the fluidics calibration feature of the C6, which will ensure that the instrument provides accurate measurement of the volume aspirated from samples.

Update Firmware: Updates the C6 firmware. Use only when directed by Accuri Cytometers to upgrade the C6 firmware with an official firmware release.

The About Menu

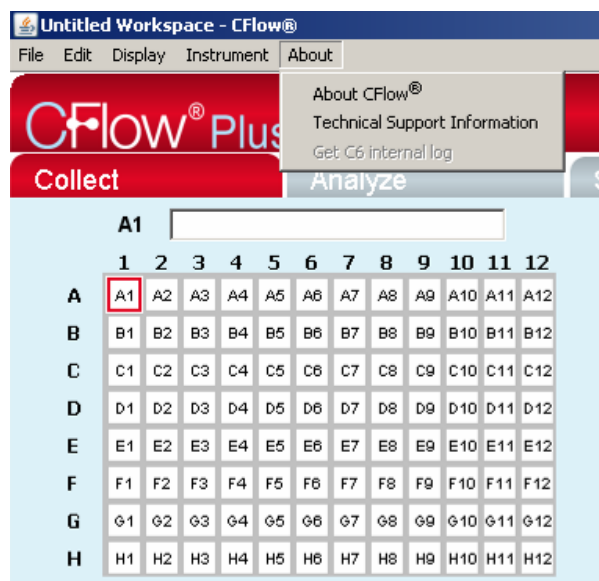


Figure 5-14 The About Menu

About CFlow: Opens a dialogue box containing the version of CFlow currently installed. It also has the contact information for Accuri Technical Support.

Technical Support Information: Opens a dialogue box containing specific information regarding CFlow and the C6. In the event of a problem with the C6, Accuri Cytometers Technical Support may ask for this information.

Get C6 Internal Log: Disabled at this time.

Chapter 6 VirtualGain

VirtualGain is a feature in CFlow that allows the mimic of detector voltage adjustments. **It is strictly an analysis tool and should not be used while collecting data.**

There are certain instances where the position of a given peak in different samples should be the same or at a specific channel number regardless of the staining. Instruments that have voltage and amp gain controls allow the “movement” of peaks from sample to sample facilitating this. CFlow has VirtualGain instead of these controls. This tool allows you to mimic voltage and amp gain adjustments to reposition data on the axis after the data has been collected. VirtualGain is a gross adjustment, an approximate visual shift of the data.

In Figure 6-1 the negative peak in the Control and Sample 1 fall in similar channels (MFI = 28.2 and 29.7, respectively). However, the negative population in Sample 2 is further to the right (MFI = 73.4). In order to align the negative population peak of Sample 2 with the Control, use VirtualGain.

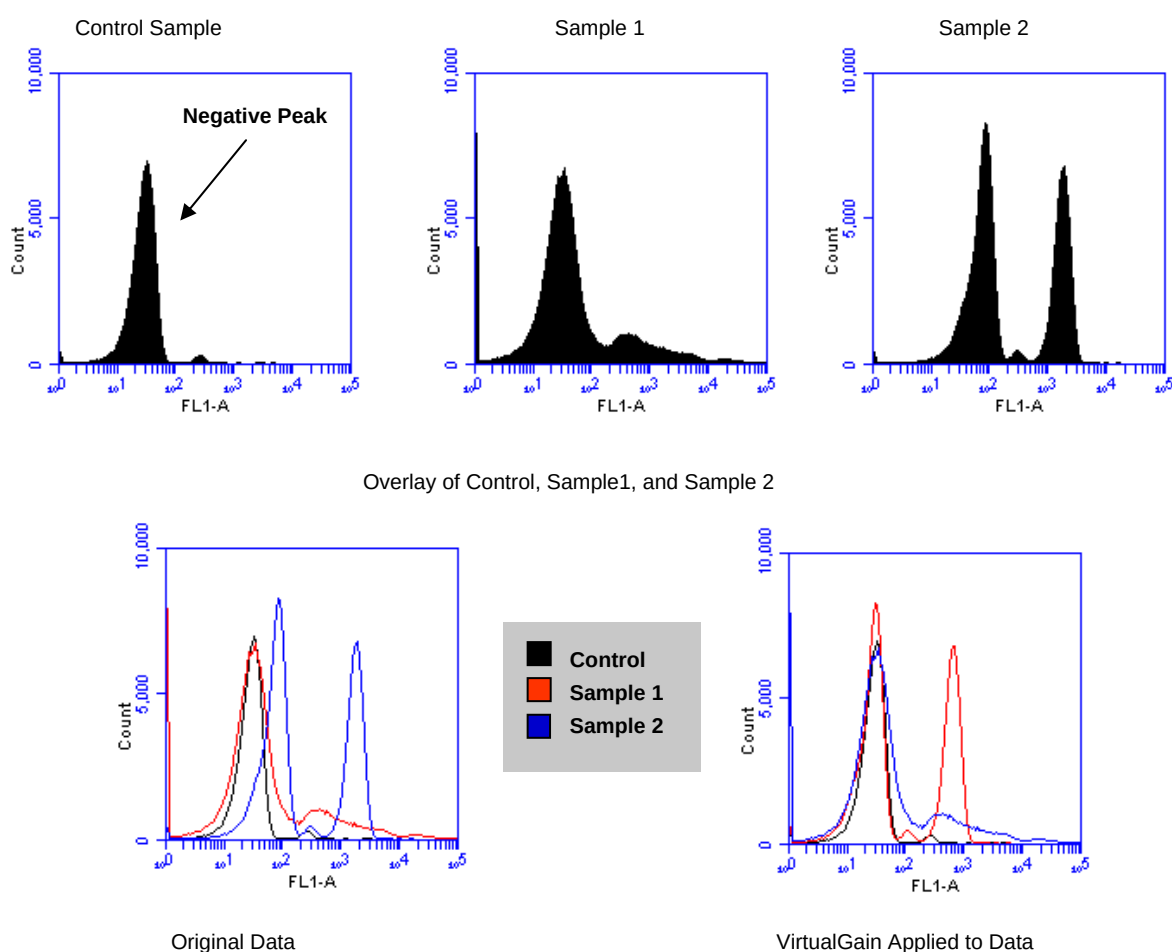


Figure 6-1 When to use VirtualGain

Applying VirtualGain

1. Open CFlow.
2. Select the Analyze tab.
3. Recreate the histogram or copy plots from Collect for application of VirtualGain (Figure 6-2).

VirtualGain can only be applied to one parameter at a time and must be done on a histogram plot. After VirtualGain is applied, the data can be viewed with or without the VirtualGain in any type of plot.

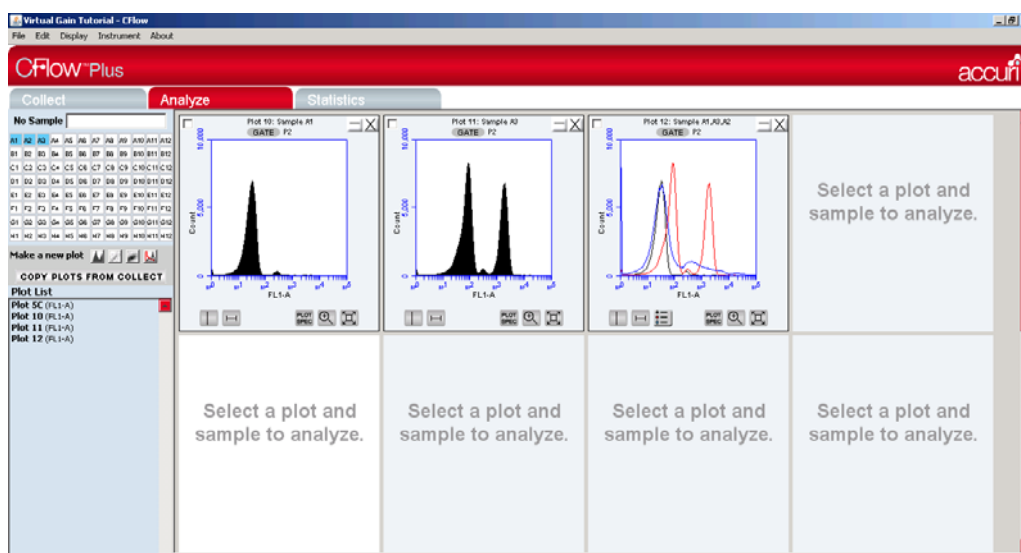


Figure 6-2 Recreate the histogram or copy plots from Collect

4. Apply the appropriate gating to the plots in Analyze. Gating is not automatically carried over in plots copied from the Collect tab.
5. Click on the histogram parameter label to reveal the **Parameter List** (Figure 6-3).

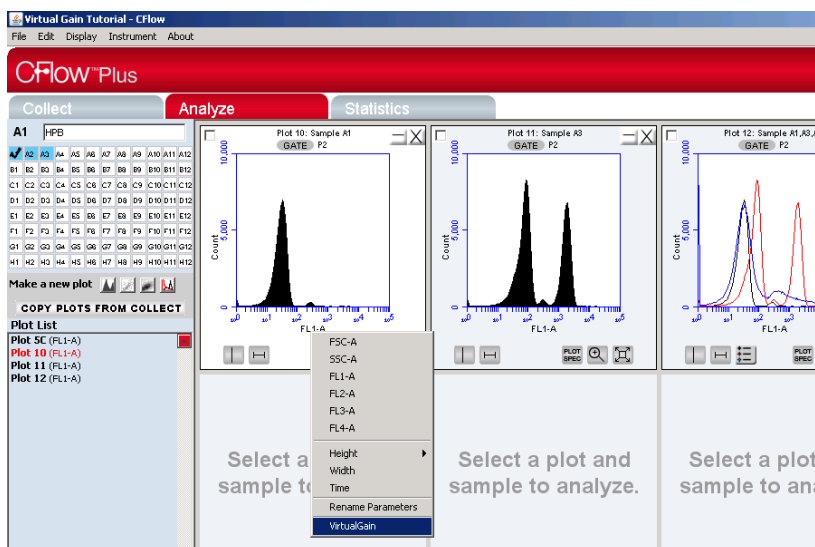


Figure 6-3 Click on the histogram parameter label to reveal the Parameter list.

6. Select **VirtualGain** from the Parameter List. The VirtualGain dialogue box will open (Figure 6-4).
7. Move the Peak Definition Marker (light blue vertical line) in the Standard Sample plot to the center of the peak that will be the reference point. Other sample(s) will be aligned to this position.

The plots in the VirtualGain dialogue box will be Zoomed to the same axes ranges as they are displayed in the Analyze tab. To Zoom in further or scale over a different range, use the Zoom Tools in the Analyze plot. There are no Zoom Tools in the VirtualGain dialogue box.

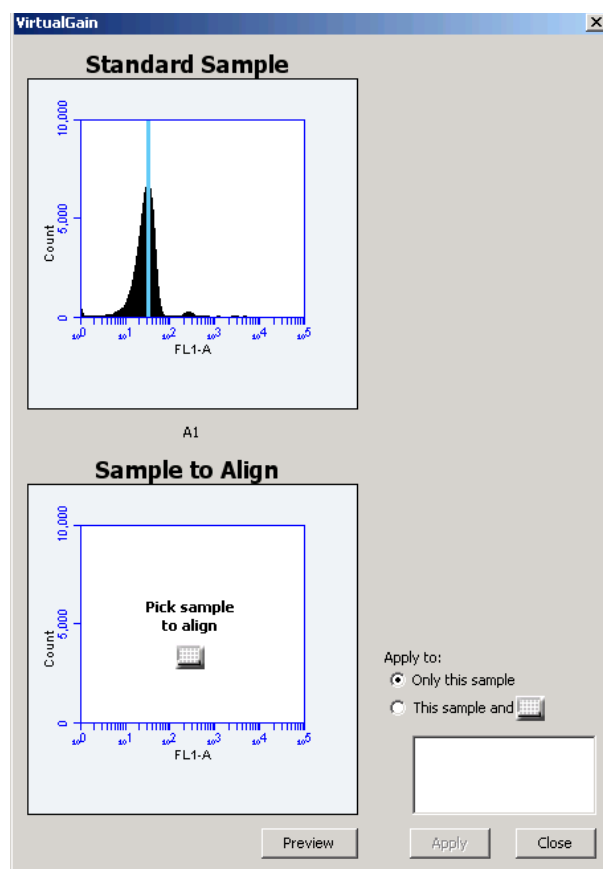


Figure 6-4 Step 7 and Step 8, Aligning plots.

8. Click on the small Sample Grid in the center of the Sample to Align plot. A larger Sample Grid will appear.

9. Pick the sample to Align (to adjust the peak position in) by clicking its well (Figure 6-5). The grey well indicates the Standard Sample already chosen. Choose from blue wells for samples to Align to the Standard. Only a single sample can be chosen to align. To add additional samples see Step 14.

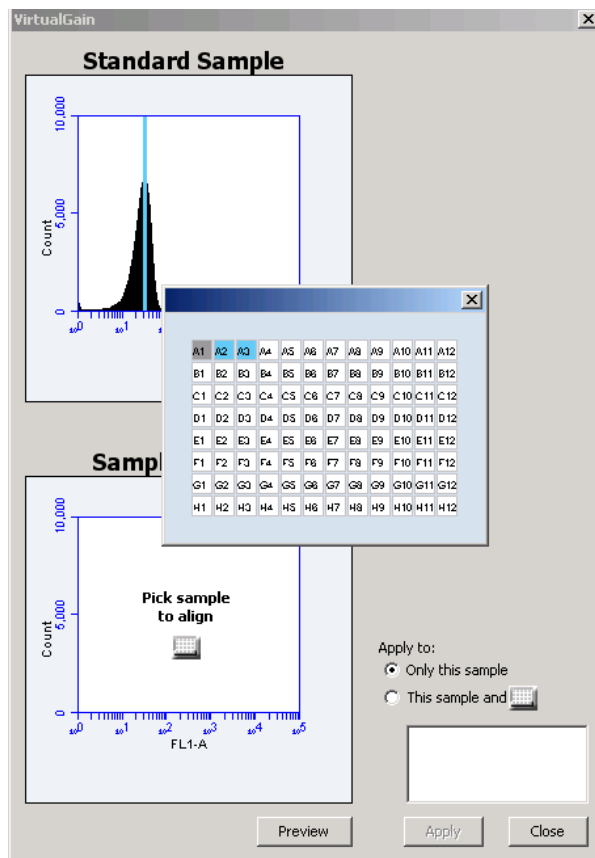


Figure 6-5 Step 9, Pick the sample to align.

10. The chosen sample will now appear in the Sample to Align plot.
11. Move the Peak Definition Marker in the Sample to Align plot to the center of the peak that will be lined up to the Standard (Figure 6-6).

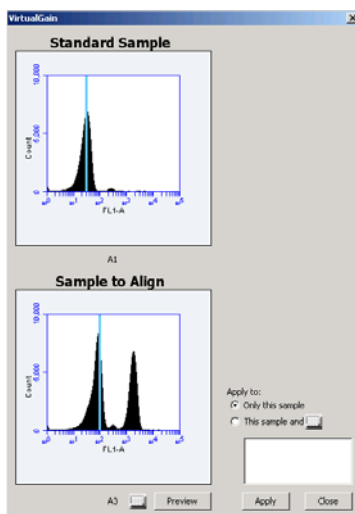


Figure 6-6 Step 11, Move the Peak Definition Marker.

12. Click **Preview**. The display will now show the aligned sample with VirtualGain applied and the peak of interest will be aligned in both plots.
13. If the sample alignment is not in the desired position, reselect the Peak Definition Marker in the Sample to Align plot and reposition it. (Repeat Steps 11 and 12.)

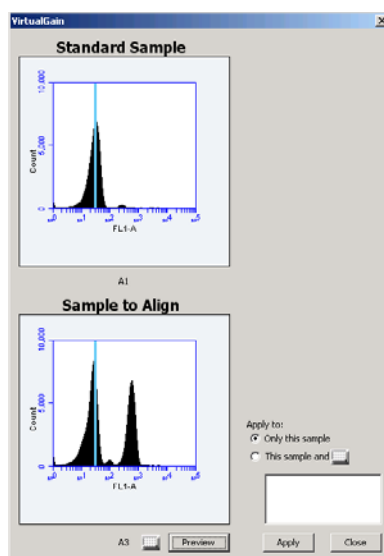


Figure 6-7 Step 14, To align other samples exactly as the first aligned sample.

14. To align other samples exactly as the first aligned sample, select “This sample and”. A sample grid will open to allow selection of additional samples. If the other samples need a different amount of VirtualGain, VirtualGain must be set individually for each one (Figure 6-7).
15. Click **Apply** to apply VirtualGain to the data. A black asterisk will now appear under the Sample to Align plot. This indicates that VirtualGain has been applied to that parameter for that sample. VirtualGain is only applied to the displayed data. It does not alter the FCS data. The VirtualGain is recorded only in the CFlow file (Figure 6-8).
16. Click **Close**.

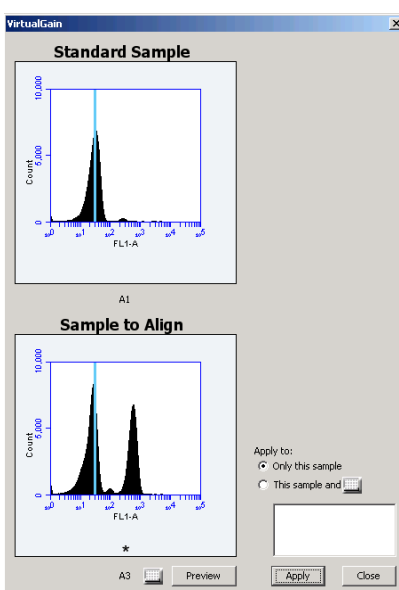


Figure 6-8 Step 15, A black asterisk appears under the Sample to Align plot.

17. The sample with VirtualGain applied will now have a black asterisk under the parameter label. The negative peak for Sample 2 now has a MFI = 26.7. Without VirtualGain, the MFI was 73.4 (Figure 5-9)

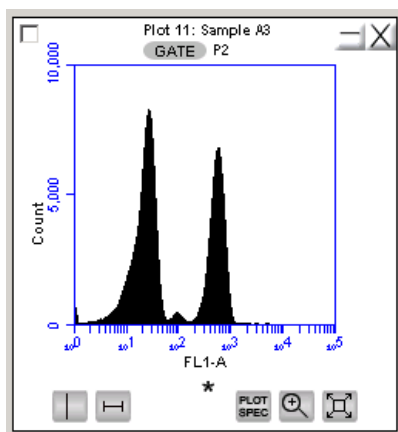


Figure 6-9 The sample with VirtualGain applied has a black asterisk under the parameter label.

18. To switch between the VirtualGain and native displays, click the asterisk. A black asterisk indicates VirtualGain applied, a grey asterisk indicates display of the original data (Figure 5-10).

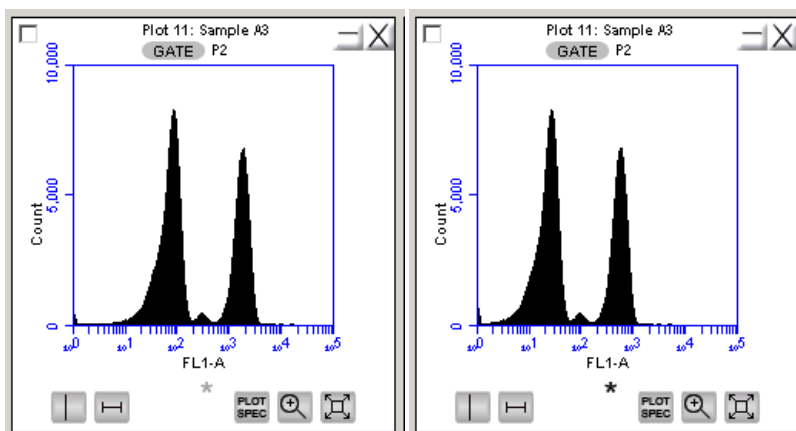


Figure 6-10 Click the asterisk to switch between VirtualGain and native displays. A grey asterisk indicated native data, a black one Virtual Gain applied.

19. Overlays will automatically display VirtualGain, when applied. Sample 2 now lines up with the Control and Sample 1. The VirtualGain asterisk is not displayed in Overlays when VirtualGain is applied to some or all of the samples in the overlay (Figure 5-11).

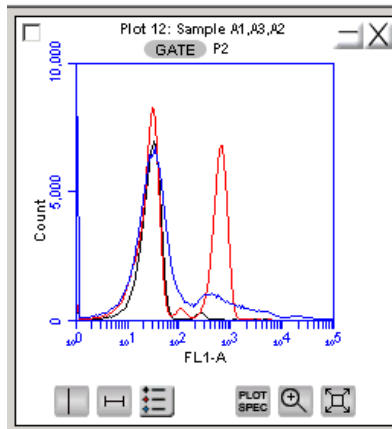


Figure 6-11 Overlays automatically display VirtualGain whenever applied.

20. To remove VirtualGain, select “**Remove All VirtualGain**” from the **Display** menu. This will remove VirtualGain from *every parameter* in *every sample* within the CFlow file.

Chapter 7 CFlow Sampler

Introduction

The CSampler™ is an optional accessory for the C6 flow cytometer that allows for the aspiration of samples prepared in 48- and 96-well plates and standard 12 x 75 mm tubes from a 24-tube rack. In order to operate the CSampler, CFlow Plus software must be upgraded to CFlow Sampler software. CFlow Sampler is based on the CFlow Plus platform, so much of the functionality is common to both platforms. This chapter outlines the additional capabilities of CFlow Sampler and differences from CFlow Plus.

Increased Data Capacity

CFlow Sampler has a higher total data capacity than CFlow Plus. When a 96-well plate is selected, the total event capacity has been increased to 96 million events. Each sample well is still limited to 1,000,000 events. The total event capacity has been similarly increased to 48 million events for a 48-well plate and 24 million events when utilizing the 24-tube rack.

Data Collection Tabs

CFlow Sampler has two tabs that can be used during data collection, Manual Collect and Auto Collect.

The Manual Collect tab is designed for running individual samples from a plate or tube rack. It can also be used to define the parameters which are going to be used to acquire data, prior to data collection, for either collection mode. The Manual Collect tab is also used to re-collect data from wells that have been previously run from the Auto Collect tab.

The Auto Collect tab is designed to automatically collect data from multiple samples in microplates or tube rack

Auto-save

CFlow Sampler automatically saves sample data after it is acquired. Auto-save will occur at the completion of data acquisition in each well, whether using the Manual Collect tab or the Auto Collect tab. Auto-save will only save the most recent data collected. (If changes are made during analysis of previously collected data, such as changing the threshold value, these changes are not automatically saved. In addition, changes to analysis must be saved using the **Save** command from the File menu.)

Export Sample Settings

Accessible from the **File** menu, sample settings can be exported as a .csv file, allowing information to be opened in spreadsheet programs. Sample settings include acquisition criteria, sample names, parameter names, and compensation values.

CFlow Plus Compatibility

CFlow Sampler files are compatible with CFlow Plus software. CFlow Sampler files utilize the same .c6 extension. All data and plots will be recreated in CFlow Plus. While sample settings such as Wash and Agitate will not be accessible, they are not lost if files are opened in CFlow Plus and then reopened in CFlow Sampler.

Choosing a Plate Type

The first action that should be performed when setting up an experiment is to select a plate type, as shown in Figure 7-1. Every time a new CFlow Sampler workspace is opened, a plate type must be selected.

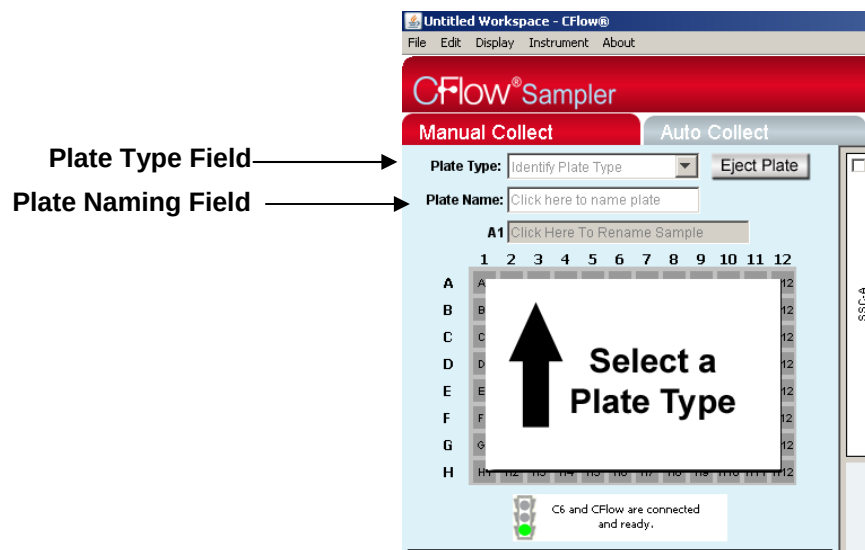


Figure 7-1 CFlowSampler start-up screen.

To assign a plate type, select the **Plate Type Menu** by clicking on the **Plate Type Field**. A drop-down menu will appear with the options, 96-well plate, 96-well (deep well) plate, 48-well plate and 24-tube rack (Figure 7-2).

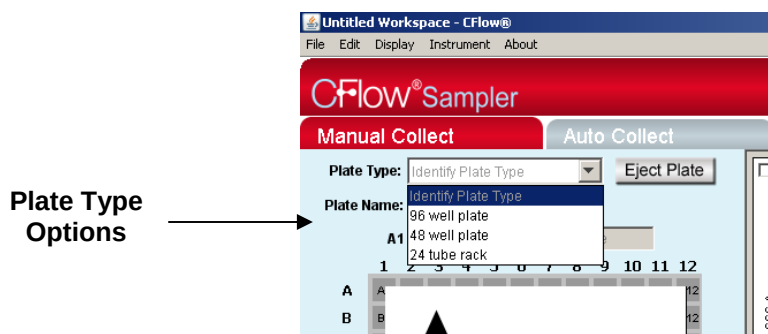


Figure 7-2 The Plate Type drop-down menu.

The Accuri CSampler is compatible with 96-well plates (standard flat, u-, and v-bottom), 96-well deep well plates, 48-well plates and standard 12 x 75 mm tubes in a 24-tube rack.

Once a plate type is selected, the sample grid will display the available wells. For a 96-well plate, all 96 wells are available, for a 48-well plate, wells A1-F8 are available, and for the 24-tube rack, wells A1-D6 are available for data collection. Data can be collected into any well and in any order using the Manual Collect tab. When using the Auto Collect tab, data can be collected either horizontally or vertically through the available wells, starting at well A1.

Naming a Plate

In addition to naming individual samples/wells within the plate, the entire plate can also be named for

future reference. Simply, type a label in the **Plate Naming Field** (Figure 7-1).

The Manual Collect Tab

The Manual Collect tab has a similar layout to the Collect tab in CFlow Plus (Figure 7-3) and is used in the same manner. Simply select a well, as indicated by the red outline, input the desired acquisition parameters, and click **RUN**. See Chapter 3 for more details.

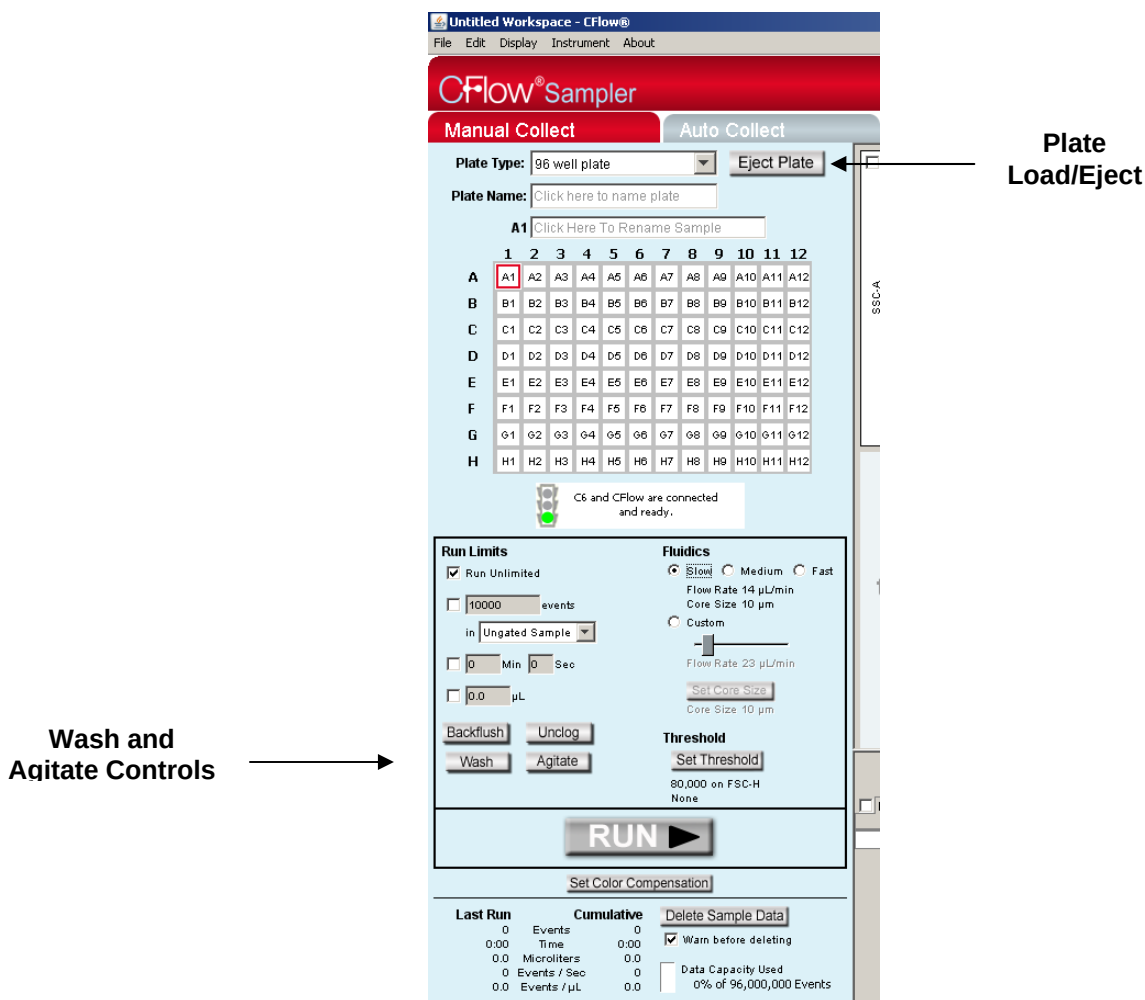


Figure 7-3 The CFlowSampler Manual Collect Tab.

There are several unique features in the Manual Collect tab: the ability to load and eject a plate, the option to wash the SIP between samples, and the ability to agitate, or mix, samples.

Load/Eject

Selecting the **Load/Eject** function will move the CSampler arm and plate platform in or out of the C6 to facilitate the placement of a plate or tube rack on the plate platform or remove a plate after sample acquisition is complete (Figure 7-4). After 15 minutes in the eject position, the arm and plate platform will automatically return to the home position with the SIP in the wash station.

Wash

Clicking the **Wash** button will activate a single wash cycle, thoroughly cleaning the SIP of any previous

sample. The contents of the SIP are aspirated up and out of the SIP, then the SIP and wash station are rinsed with clean sheath fluid. Selecting Wash from the Manual Collect tab will perform a single wash cycle. Using Wash from the Auto Collect Tab allows for multiple washes to be performed automatically after each well is complete. Up to 5 wash cycles can be selected. Experience has shown that running one wash cycle will typically reduce carry-over to <1.0%. Running two wash cycles will typically reduce carry-over to <0.1%.

Agitate

Clicking the **Agitate** button will activate a single agitation cycle. Agitate is designed to keep a sample in suspension. It is not designed to re-suspend a completely settled sample. Selecting Agitate from the Manual Collect tab will perform a single agitation cycle. Using Agitate from the Auto Collect tab allows for multiple cycles (up to 5) to be performed automatically at given time intervals during the plate run. Agitation is only performed when the SIP is clear of any well or tube.

Fluidics Commands

Since the SIP is not easily accessible with the CSampler installed, some of the fluidics commands have been modified.

Backflush

When the **Backflush** command is selected, the CSampler will automatically move the arm and plate platform to the Eject position, allowing a container to be placed under the SIP. After placing a container under the SIP to catch any fluids that will be ejected from the SIP, click the **Backflush** button in the Run Backflush Cycle window (Figure 7-4).

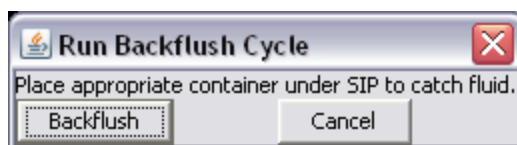


Figure 7-4 The Run Backflush Cycle window.

Unclog

When the **Unclog** command is selected, the CSampler will automatically move the arm and plate platform to Eject position. After placing a container under the SIP to catch any fluids that will be ejected from the SIP, click the **Unclog** button in the Run Unclog Cycle window (Figure 7-5).

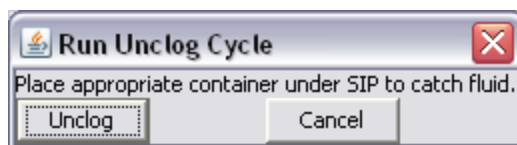


Figure 7-5 The Run Unclog Cycle window

Calibrate Fluidics

The fluidics of the C6 can be calibrated for the volumes typically run from a 96- well plate. This operation requires that at least 150 μ L is placed within the well. Once a plate is prepared with the appropriate volume, load it onto the CSampler. Upon selecting Calibrate Fluidics, a window will indicate which well the C6 is going to use for the fluidics calibration (Figure 7-6). This well will also be indicated by a red box in the Sample Grid. If this is not the desired well, switch to the Manual Collect tab and select the proper

well. After the proper well is indicated, click Calibrate. The calibration will take several minutes. Refer to the Accuri C6 Instrument Manual for detailed information.

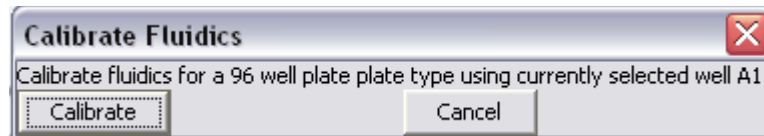


Figure 7-6 The Calibrate Fluidics window.

Using the Manual Collect Tab

The Manual Collect tab is designed for three purposes:

1. To run samples individually from any plate type or tube rack. When using this tab, settings such as Run Limits, Thresholds, and Color Compensation can be applied to either the current sample being viewed, indicated by a red box surrounding the well, or to all the samples in the sample grid.
2. To assist in setting up experiments that will be processed using the Auto Collect Tab. The following can be done using the Manual Collect Tab, prior to swapping to the Auto Collect Tab:
 - a. Plot creation
 - b. Gating schemes
 - c. Setting threshold values, primary threshold channel (trigger), secondary threshold channel
 - d. Color compensation
 - e. Run Limit settings applied to individual wells from the Manual Collect tab are not carried over into the Auto Collect tab.
3. The Manual Collect tab can also be used to collect data from a plate if the plate has been interrupted or aborted in the Auto Collect Tab.

All of the plot tools available in CFlow Plus are available in the Manual Collect tab. Creating plots, regions, gating schemes, and other analysis features are described in Chapter 3.

The Auto Collect Tab

The Auto Collect tab (Figure 7-7) is designed to facilitate the automatic running of multiple samples from a well plate or the sample tube rack.

There are two fundamental differences between the Manual Collect tab and the Auto Collect tab. The first is that the Auto Collect tab does not allow the creation of plots. This has been replaced with the ability to easily label a large number of samples. The second difference is the ability to create “sets” of samples.

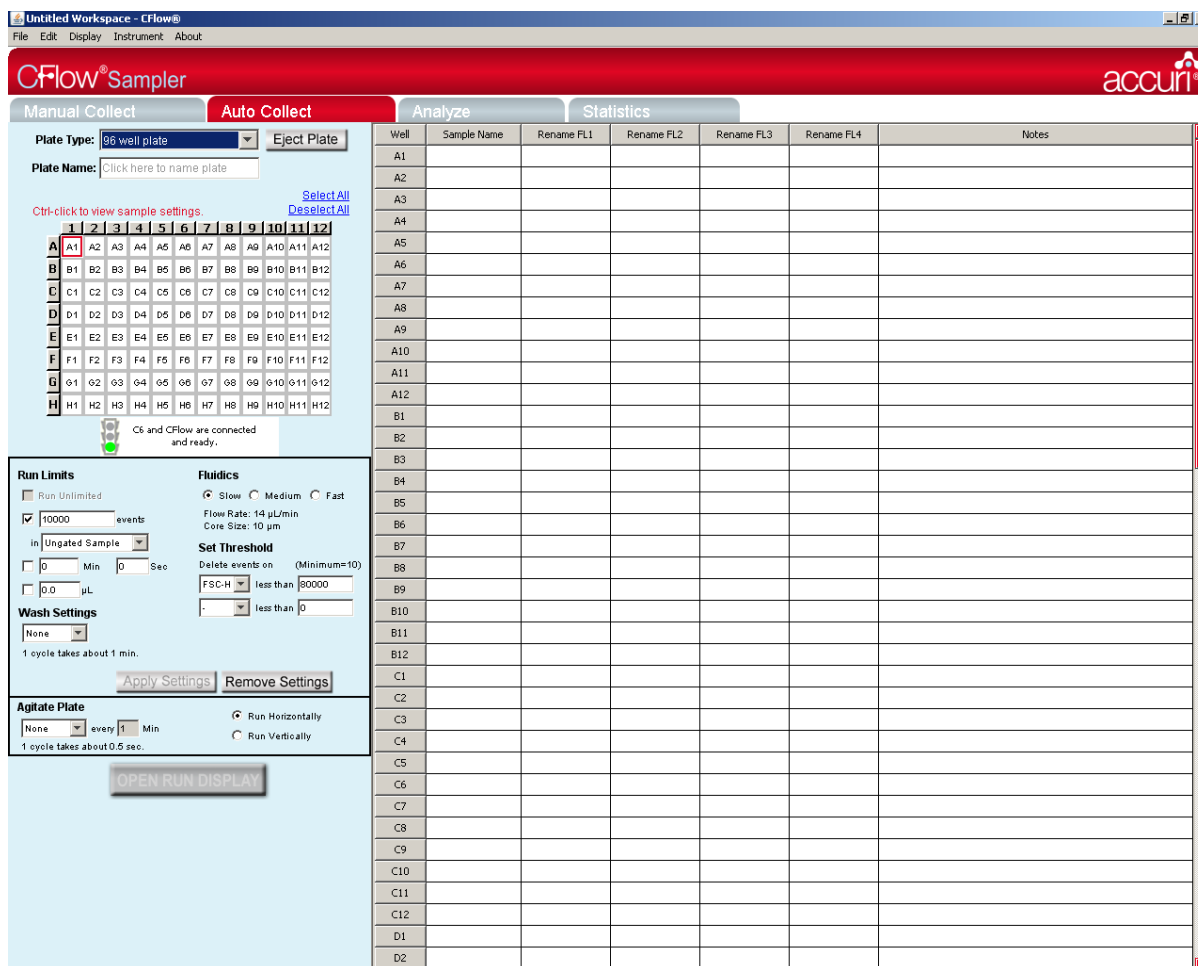


Figure 7-7 The Auto Collect tab

Sample Annotation Table

The right side of the Auto Collect tab contains the Sample Annotation Table. This table allows for the naming of samples, renaming of parameters, and adding a specific notation to each sample. Information can be entered manually for each individual sample or directly imported from a spreadsheet program using the Copy (Ctrl+C) and Paste (Ctrl+V) commands. The table layout is fixed and therefore sample names must be entered across rows (1-12) before moving down the columns (A-H) of the sample grid.

Individual fields can be selected by clicking in them with the mouse or advancing with the Tab key. The keyboard arrow keys do not function in this view.

Text in a field can be select by double clicking in the field.

When the **Open Run Display** button is selected, the Sample Annotation Table is replaced with a new

window containing a single plot. This plot is described in the Run Display section.

Creating Sample Sets and Applying Acquisition Settings

Selecting Samples

A sample well can be selected by clicking on it with the mouse. Unlike the Manual Collect tab where selected wells are indicated by a red box, selected wells in the Auto Collect tab will contain a black checkmark (tick) as seen in Figure 7-8.

Creating a “Set” of Wells (Samples)

A set is a grouping of samples that will be acquired using the same acquisition criteria (number of events, threshold setting, etc). Sets can be created in several ways:

1. Individually select the wells.
2. Click on the Column (1-12) or Row Header Labels (A-H). This will select the entire column or row. Multiple rows and columns can be selected simultaneously.
3. Selecting all the sample wells in the Sample Grid. Above the Sample Grid are “Select All” and “Deselect All” commands (in blue text). Clicking “Select All” will select all the wells.

Samples can be unselected by using the same methods.

An entire set can be selected by double-clicking on any of the sample wells within that set.

Once single or multiple wells have been selected, settings can be applied. This is indicated by the **Apply Settings** button becoming active (Figure 7-8).

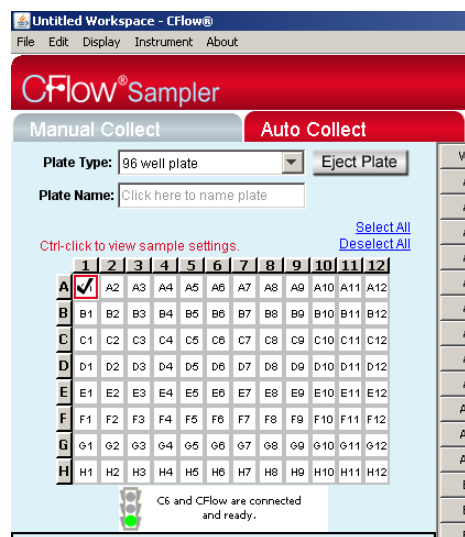


Figure 7-8 Selecting an individual sample.

Apply Settings

After a sample or set of samples has been selected, input the criteria by which these samples are to be collected. This includes Run Limits, Fluidic settings (Slow, Med, Fast), and the desired threshold(s). The option for custom fluidic settings (Flow Rate and Core Size) has been disabled in the Auto Collect tab. Wash cycles (0-5) can be chosen to occur after each sample well is complete. Apply settings by clicking the **Apply Settings** button. Selected samples will be highlighted the same color once settings have been applied to the set, or group of samples (Figure 7-9).

If Run Limit or Flow Rate and Core Size settings are modified using the Manual Collect tab, those settings will not change in the Auto Collect tab. Threshold settings and Color Compensation settings will apply to both tabs.

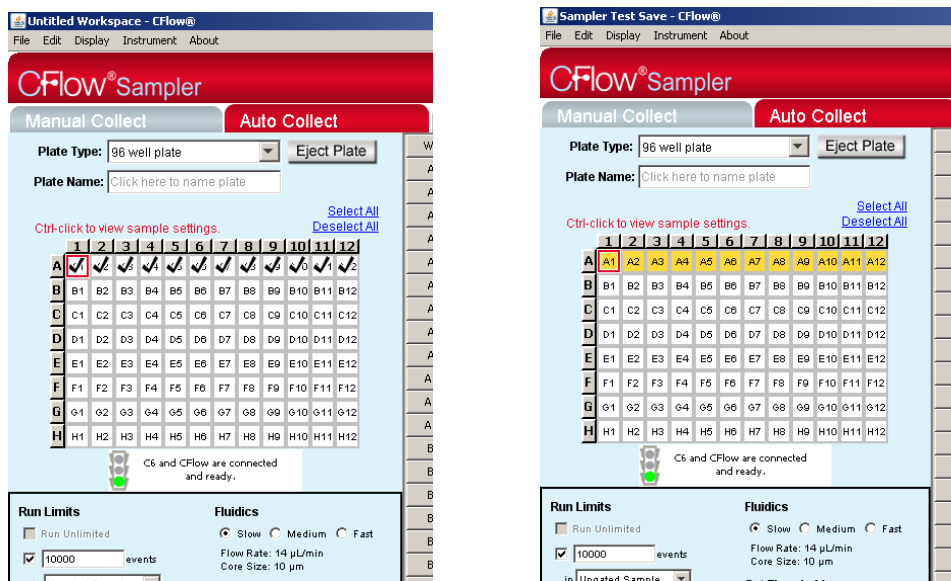


Figure 7-9 Applying settings to a set of samples will turn those sample wells the same color.

Each new set of samples or sample settings will be displayed with a different color (Figure 7-10). There are 12 colors available; however, up to 96 sets can be created. The colors will be reused in the same order starting with the 13th set created.

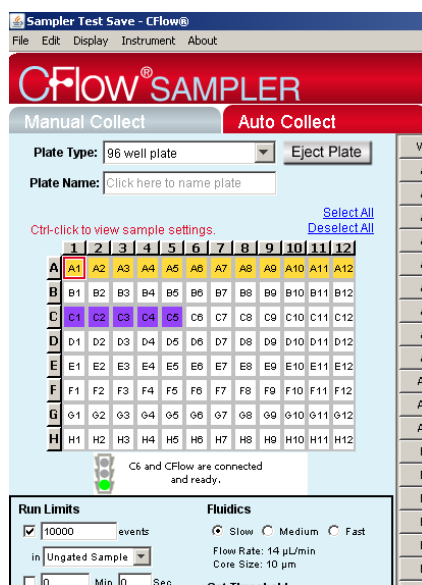


Figure 7-10 The Auto Collect tab displaying two sets.

The first time **Apply Settings** is selected, CSampler will prompt a Save File dialogue box. This will designate the name and location of the file that will contain the data about to be run in this session of

CSampler. This is where Autosaved data will be saved.

Once the file has been saved, the **OPEN RUN DISPLAY** button will become active.

Every time **Apply Settings** is clicked while defining the acquisition parameters for the plate, the settings are resaved. Once the Auto Collect Run has been started, the data will be automatically saved after each sample is complete and before the next well begins to acquire data.

Modifying Settings

After a set is created, its settings can be modifying by reselecting the well that are to be changed. After changes are made and settings applied, selected wells will change to a new color.

View Sample Settings

After settings have been applied to a workspace, the settings of a particular well can be viewed by Ctrl+Click on the well of interest. The red box will move to the well that has been selected this way.

Remove Settings

After settings have been applied to a set, they can be removed using the **Remove Settings** button. Settings can be removed from one or all of the samples within the set by either reselecting the entire set (double-clicking one of the samples within the set) or selecting the samples individually by clicking on them. When settings are removed from a sample, the sample well will revert to a white box. The acquisition settings displayed in the Control Panel will not change.

Agitate

While running from Auto Collect, CSampler can automatically perform plate agitation at periodic intervals. Agitation can be set to occur in 1 minute intervals, up to 30 minutes, throughout the duration of the plate run. Agitate will not interrupt data collection. If the interval is reached during acquire, the well will finish and then the agitation will occur. As stated previously Agitate can be selected to perform between 0 and 10 agitate cycles.

Agitate settings are applied to the entire plate or tube rack and can not be applied to individual wells.

Run Direction

Data can be collected from plates in either the horizontal (A1, A2, A3, etc.) direction or the vertical (A1, B1, C1, etc.) direction. This is determined by choosing the **Run Horizontally** or **Run Vertically** toggle beneath the Settings Controls in the Control Panel.

The Run Display

The Run Display is used during data collection in the Auto Collect tab. To access the Run Display window, click the **OPEN RUN DISPLAY** button. This button is only active after one or more wells have had settings applied. Once the Run Display is opened the Sample Notation Table will be replaced with the Run Display. The Run Display contains a single larger plot (the Sample View Plot), an additional traffic light status indicator, the data acquisition counters, and several Pause alternatives (Figure 7-11).

The Control Panel is disabled while the Run Display is open. In order to return access to the Control Panel, the Run Display must be closed using the **CLOSE RUN DISPLAY** button.

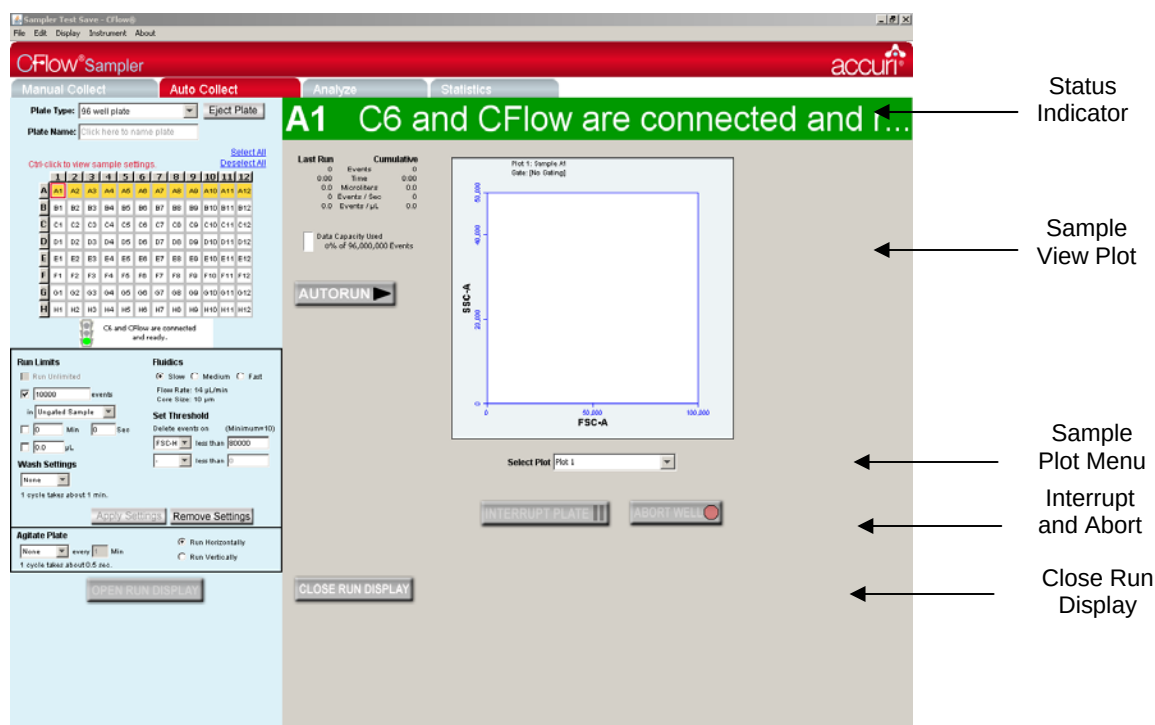


Figure 7-11 The Run Display

Sample View Plot

All plots created in the Manual Collect tab are listed in the Select Plot drop-down menu beneath the plot window and can be selected at any time during data collection. One plot at a time can be viewed. Plots can only be modified, deleted or added from the Manual Collect tab.

Alternative Pause Commands

In place of the traditional **PAUSE** button found in CFlow Plus, CFlow Sampler contains an **INTERRUPT PLATE** button and an **ABORT WELL** button. These buttons will only be active when CFlow Sampler is acquiring data.

INTERRUPT PLATE will complete data collection from the current well and perform specified wash and agitate cycles before stopping the plate run. At this point, the plate can be ejected. When a plate is interrupted, the Auto Collect tab is disabled, except to restart, and the settings cannot be changed. In order to collect data during an Interrupt state, the Manual Collect tab must be used. This ensures that the run settings of the interrupted plate are retained when the plate run is restarted. The plate run can be

restarted by selecting the AutoRun button. Upon restart of the plate, CFlow Sampler will begin at the next sample well.

ABORT WELL will immediately stop data acquisition, stop the fluidics, and eject the plate. This is similar to **PAUSE** in CFlowPlus. Upon restart of the Run, CFlowSampler will begin at the next well. It will not continue to collect from the aborted well. Similar to **INTERRUPT PLATE**, the Auto Collect tab is disabled after an aborted well. Settings cannot be changed from the Auto Collect tab in the aborted state.

Acquiring Data

To begin data acquisition, click the **AUTORUN** button.

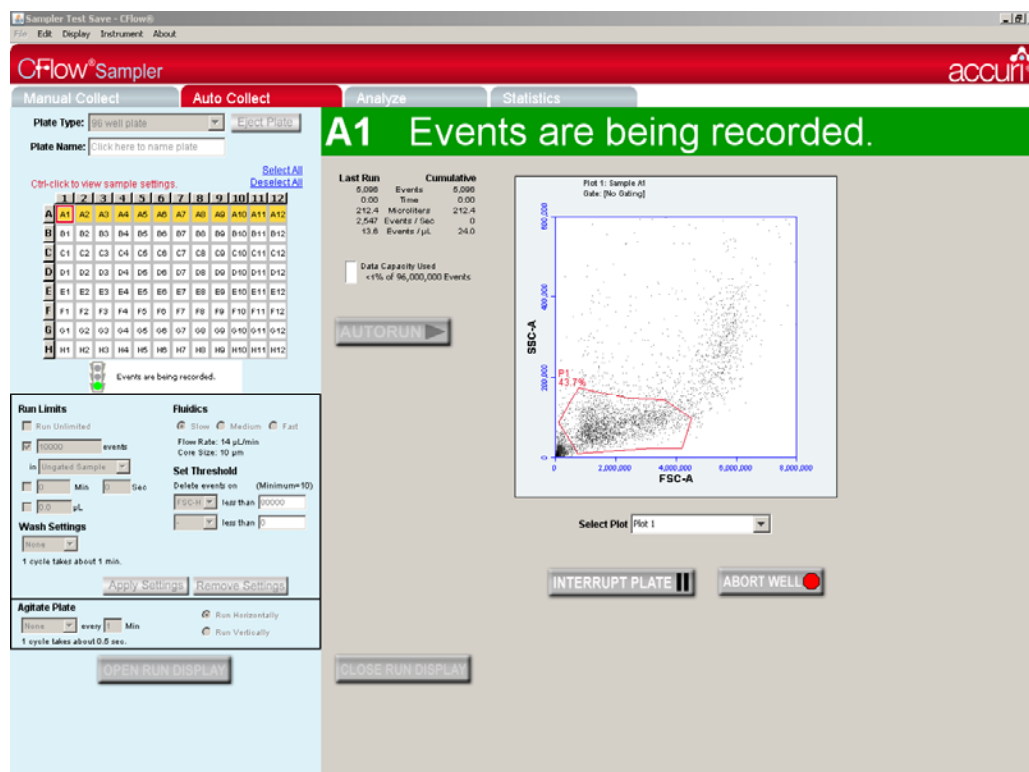


Figure 7-12 The Run Display while collecting data.

Within Auto Collect, CSampler will always start collecting from position A1. If A1 is not selected as part of a set, CSampler will advance either horizontally or vertically to the first well that has settings applied to it and begin acquiring data.

As data is acquired and the CSampler advances through the plate or tube rack, completed samples will display a small blue square in the upper left corner of the well. The red box indicates which sample the CSampler is currently acquiring data from. The current well will also flash blue during data acquisition, similar to CFlow Plus.

When the plate or all of the sets of samples have finished, **DONE!** will be prominently displayed across the Run Display window (Figure 7-13).

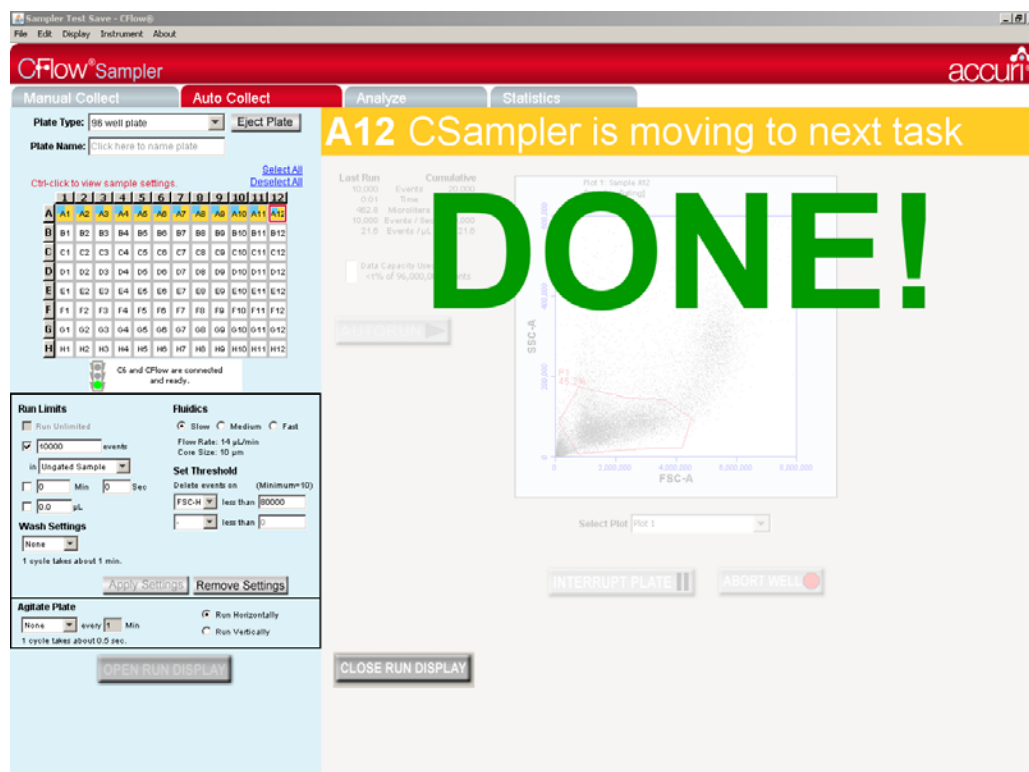


Figure 7-13 The CSampler display after completion of a sample run.

Once a plate is complete, the Run Display must be closed in order to continue using CFlow Sampler. The Run Display is closed by clicking the **CLOSE RUN DISPLAY** button. This will return the Sample Annotation Table to the screen. Analysis can then be completed using the Analysis and Statistics tabs.

Analyze and Statistics Tabs

The Analyze and Statistics tabs are used in the same manner as CFlow Plus. Please see Chapter 3 for a complete description of these tabs.

Running a Sample Plate (Example)

The following is intended to provide an example of a typical setup and data collection workflow using CFlowSampler software.

1. Open CFlow Sampler software.
2. Select the Manual Collect tab.
3. Select a plate type from the Plate Type Menu.
4. Create the plots that are going to be needed for running the plate or load the template.
5. If necessary, acquire some events to define regions, gating, etc.
6. Switch to the Auto Collect tab.
7. Select the wells in the Sample Grid where data will be collected.
8. Set the desired acquisition settings, such as number of events, threshold, washes needed, etc.
9. Apply Settings.
10. Define the file name for Autosave.
11. Enter sample names and labels into the Sample Annotation Table manually or import them from a spreadsheet program.
12. Select agitation schedule.
13. Select the direction the plate is to be Run.
14. Open the Run Display
15. Select the Plot to view during acquisition from the Plot dropdown
16. Click AutoRun.
17. When the plate is complete, close the Run Display.
18. Switch to either the Manual Collect or Analyze tabs to review and/or analyze the data.

Collisions and Alignment

If instructed to do so by the software, an alignment should be performed by selecting **Align** from the popup (Fig. 7-14). Alignment can also be performed at any time by selecting **Align** from the **Instrument** Menu.

If there is an obstruction in the path of the CSampler arm, CFlowSampler will indicate that a collision has occurred. The message box shown in Figure 7-14 will be displayed, together with a red Traffic Light. In order to continue using the CSampler, an alignment must be performed. Before running an alignment any objects previously placed on the CSampler mat should be removed.

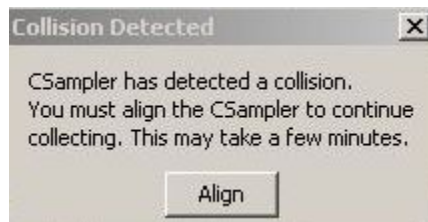


Figure 7-14 Collision detected window

If a second collision occurs a second alignment will begin automatically. If the secondary alignment fails, please contact Accuri Technical Support.

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